

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant(s): Rosen et al.	
Application No.: 09/960,665	
Filed: 9/21/2001	Group Art Unit: 1624
Title: Methods and Compositions for Degradation and/or Inhibition of HER Family Tyrosine Kinases	Examiner: Bruck Kifle
Attorney Docket No.: MSK.P-038-2	

**BRIEF FOR APPELLANT**

This brief is filed in support of Applicants' Appeal from the final rejection mailed 12/30/2005. Consideration of the application and reversal of the rejections are respectfully urged.

**Real Party in Interest**

The real party in interest is the assignee, Sloan-Kettering Institute for Cancer Research. The application is licensed to Biogen Idec Inc..

**Related Appeals and Interferences**

This case is related to US Patent Application No. 09/937,192 which was the subject of Appeal 2006-1468, decided August 28, 2006. As discussed below, many of the issues in the case are the same as in that case, and a copy of the decision in that case reversing the rejections is attached in the appendix.

### Status of Claims

Claims 35-66 are pending in this application and are the subject of this appeal. Claims 1-34 have been canceled. No other claims have been presented in this application.

### Status of Amendments

No amendment after final rejection was filed.

### Summary of Claimed Subject Matter

The present application relates to chemical compounds and to methods of using those compounds. As set forth in independent claim 35, the chemical compound of the invention comprises "first and second hsp-binding moieties which bind to the pocket of hsp90 with which ansamycin antibiotics bind." (Page 4, lines 9-13) These binding moieties are connected to one another by a linker and each retain the ability in the chemical compound to bind to the pocket of hsp90. (Page 4, line 14) Thus, the composition of the invention is essentially a dimer (homodimer or heterodimer) formed from compounds that bind to hsp90 as monomers and that retain this ability in the compound of the invention. (Page 4, lines 20-28).

Independent claims 44 and 53 recite methods of using the compounds as set forth in claim 35. Claim 44 refers to a "method for destruction of cells expressing a HER-family tyrosine kinase" by administering such a compound to the cells. This is the same activity observed for the monomeric compounds, but compounds of the invention are observed to have greater selectivity with respect to the kinases they inhibit than the corresponding monomeric compounds. (Page 4, lines 29 -31; Page 6, Table 1 and lines 23-30) Claim 53 refers to "a method for treating cancer in a patient suffering from cancer" by administration of the compound of the claim 35.

### Grounds of Rejection to be reviewed on Appeal

1. Claims 35-66 stand rejected under 35 USC § 112, first paragraph for lack of enablement.
2. Claims 35-66 stand rejected under 35 USC § 112, second paragraph, as indefinite.

The claims are also provisionally rejected for obviousness-type double patenting. As in the related case (SN 09/937,192), this issue is not contested, and a terminal disclaimer will be filed in the later of the two cases to reach allowance.

## Argument

### Enablement Rejections

#### A. Composition claims 35-43

Claims 35-43 stand rejected under 35 USC § 112, first paragraph, as lacking enablement. The Examiner asserts that the enablement of the specification is limited to geldanamycin dimers, with certain linkers, linked at certain positions.<sup>1</sup> In support of this rejection, the Examiner presented an argument that was in all material respects identical to the argument that was found lacking in Appeal 2006-1468 where the Board of Appeals found that the Examiner had failed to present a *prima facie* case of unpatentability under 35 USC § 112, first paragraph. Thus, on this basis, the rejection should be reversed.

Applicants note that the scope of the composition claims in this application is different from the scope of the composition claims in Appeal 2006-1468. Specifically, in the prior case, the composition is a dimer of ansamycin antibiotics that retain the ability to bind to the pocket of hsp90 to which monomeric ansamycin antibiotics bind. In the present case, the two parts of the dimer are defined more generically as a hsp-binding moieties which bind to these pockets and which, in the dimeric form retain the ability of the monomeric units to interact with hsp90 and cause the same result (degradation of proteasomes). The Examiner has presented no arguments

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<sup>1</sup> The Examiner generalized the enablement rejection to include both composition and method claims, and included in the statement of the rejection that the claims are only enabled for the treatment of breast cancer when the linker was (CH<sub>2</sub>)<sub>4-12</sub> and Her-2 expressing cancer with the linker was a 4-carbon chain. As these bases for the rejection are not applicable to the composition claims, the two sets of claims are argued separately.

with respect to this claim scope that are distinct from those present in the related case with the claims limited to ansamycin antibiotics. Indeed, the substantial focus of the rejection is on the enablement for multiple types of cancers, which is not relevant to a composition claim which would be patentable if enablement (and thus utility) were shown for a single type of cancer or for some other purpose.

The Examiner does observe in Office Action of December 30, 2005, that "the compounds embraced by the claims do not give reasonable assurance that all or substantially all of them would work." It is noted that the specification asserts that they will work, and that cases such as *In re Marzocchi* 439 F.2d 220, 223-4, 169 USPQ 367, 369-70 (CCPA 1971) place the burden on the Examiner to explain why this assertion is not believable through the presentation of acceptable evidence or reasoning. This the Examiner has not done. Furthermore, since the claims are directed to compositions that are dimers of compounds that initially as monomers have a certain property (binding to hsp90) and that retain that property in the dimeric compound, it does not seem that a reasonable basis for doubt can be presented.

It is further noted that case law makes it clear that enablement is not required for things that have not yet been invented. The Examiner states that "there is no allegation that Applicants enable future inventions. The problem with the claim is determining the compound claimed." (Office Action of December 30, 2005, Page 2.) From this statement, it appears that the Examiner is confusing the first and second paragraphs of § 112. In any event, the Examiner has not identified any reasons why a person skilled in the art could not make dimers of compounds that bind to the binding pocket of hsp90.

**B. Method Claims 44-52**

These claims relate to the destruction of cells that express a HER-family tyrosine kinase. Claims 44-52 have a structure similar to claim 12 in the related case. As in the related case, the Examiner has not addressed these claims separately from the method of treatment claims. As

such, most of the arguments presented that relate to treatment of different types of cancer are irrelevant, since cancer is not mentioned in the claims.

One comment directed specifically to these claims is set forth on Page 6 of the Official Action of December 30, 2005, where the Examiner states that "regarding claim 44, there is no disclosed benefit in destroying all cells, including healthy ones, as claimed in claim 44." It is not clear if this supposed to be a utility based enablement rejection, but if so, it misses the point. Persons skilled in the art are presumed to know which cells they want to destroy, and to be able to use the teaching in the application to apply the method in an appropriate manner. Furthermore, claim 44 does not specify destruction of all cells, only those that express Her-family kinases, which presumably are present in combination with cells that do not express Her-family kinases.

C. Method claims 53-66.

Claims 53-66 are directed to a method for treatment of cancer. In addition to the comments on the compositions, which are addressed above, the reason for rejection of these claims is the assertion that the specification does not provide enablement for treatment of cancer generally. This same argument was made in the related case, and it was found by the Board of Appeals that a *prima facie* case for lack of enablement had not been presented.

In this case, as in the related case, Applicants have submitted exhibits consistent with the broad scope of effectiveness of the compositions of the present invention and why the scope of the claims is appropriate. Copies of these Exhibits are attached in the Appendix. In response, the Examiner has stated that the evidence is not commensurate with the scope of the claims, because the claims are directed to compositions that have two hsp-binding moieties, while the evidence shows results for compounds with only one binding moiety. Applicants submit that this argument is in error and unsupported by required reasoning or the law.

The copies of the papers submitted show that a monomeric ansamycin compound, 17-allylamino-geldanamycin (17-AAG), which is mentioned in the specification on Page 8, line 15

and other hsp90 inhibitors are efficacious in a variety of tumor types including breast cancer, ovarian cancer, pancreatic cancer and gastric cancer (the cancer types specifically mentioned on Page 8, lines 9-11 of the application), other HER kinase overexpressing tumors, and tumors which do not over express HER kinase. For example, Yang et al. (Exhibit A), report inhibition of glioma (brain tumor) cells with 17-AAG. Okabe et al. (Exhibit B) reports in vivo activity of herbimycin A (an ansamycin antibiotic) against leukemia cells. Kelland et al (Exhibit C, JNCI 91: 1940, 1999) achieved tumor cytostasis in two human colorectal carcinomas, HT29 and BE for the July 7, 2005 duration of drug treatment with 17-AAG. Burger et al (Exhibit D Proc. AACR, 41: Abstract # 2844, 2000) reported potent effects of 17-AAG against a melanoma xenograft and, interestingly, preliminary data from the London arm of the 17-AAG trial indicates that melanoma (2/6 objective responses) may be a responsive tumor (Exhibit E Banerji et al, Proc. ASCO, Abstract # 326, 2001) 17-AAG has also been used in studies with prostate cancers, and it has been shown that this administration resulted in dose-dependent inhibition of androgen-dependent and -independent prostate cancer xenografts. (Exhibit F Solit et al., Clin. Cancer Res. 8: 986-993, 2002). 17-AAG has also been shown to enhance paclitaxel-mediated cytotoxicity in lung cancer cells (Exhibit G Nguyen et al, Ann. Thorac. Surg. 72: 371-379, 2001); and to modulate metastasis phenotypes in non-small cell lung cancer (Exhibit H Nguyen et al., Ann. Thorac. Surg. 70: 1853-60, 2000). Thus, the efficacy of compounds that bind to the hsp90 receptor span a wide range of unrelated cancers, thereby refuting the Examiner's statement that generalized cancer therapy is inherently unbelievable. In the related case, the Board of Appeal commented on these references and stated that the "examiner has not brought in any evidence or provided any scientific reasoning to refute the evidence provided by Applicant." (page 6). The same is true in this case.

The basis for the Examiner's argument is largely that the Examiner is classing cancer therapy with perpetual motion machines and assumes in assessing enablement that it is inherently unbelievable that a cancer therapy could work generally. Such may have been the case when In re Buting, 163 USPQ 689 (CCPA 1969), cited by the Examiner, was decided in 1969, but the art

and the law have progressed since then. The notion of automatic unbelievability is no longer credited. Indeed, as the Board of Appeals noted in 1987 in *Ex parte Rubin*, 5 USPQ2d 1461, 1462 (POBAI 1987), "'contemporary knowledge in the art ' has far advanced since the days when the any statement of utility in treating cancer was per se 'incredible.'" Here, the Examiner has not offered any reasoning as to why the assertions of general utility in this application, given the suggested mechanism of action. As such, the Examiner has failed to meet the burden discussed in *In re Marzocchi*, 169 USPQ 367, 369 (CCPA 1971), where it is noted that:

a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond to those used in describing and defining the subject matter sought to be patents *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112, *unless* there is a reason to doubt the objective truth of the statements contained therein, which must be relied upon for an enabling disclosure.

An over thirty-year-old case, discussing the state of the art at that time, is not a reason to doubt the truth of the asserted utility here.

As observed in *In re Bowen*, 492 F. 2d 859, 181 U.S.P.Q. 48 (C.C.P.A. 1974) reasons are required to support an enablement rejection where the operability of materials beyond the scope of specific examples is challenged by the Examiner. The Examiner has provided no such reasoning here. The evidence of record shows that monomeric compositions has utility against a broad range of cancers. The Examiner does not contest this. He argues, however, that the evidence is not relevant because it not directed to the dimeric compositions of the invention. In making this argument, however, the Examiner has not provided a single reason why a person skilled in the art, would doubt that the dimers would have the same range of effectiveness and utility as the monomers. Thus, he has failed to provide an objective reason to doubt the statements of utility in the specification as required by *Marzocchi*.

### Definiteness Rejection

The Examiner has rejected claims 35-66 under 35 USC § 112, second paragraph as indefinite, citing two terms "linker" and "bind" as the basis for this rejection. Both of these terms were also said to be indefinite in the related case, and both rejections were reversed. The same should occur here.

#### A. The Term "Linker"

The Examiner rejected the claims based on the term linker, asserting that "nature of the linker is unknown." From the Examiner's recitation of possible linker options, it is apparent that the Examiner is equating breadth with lack of definiteness. Case law is clear, however, that the two are not the same. See, *In re Skoll*, 187 U.S.P.Q. 481 (C.C.P.A. 1975). The specification states that "the two hsp-binding moieties are joined by a linker." (Page 4, lines 14-15). Thus, the recitation of a linker in the claims requires that there be something in between the binding moieties. The person skilled in the art would have no difficulty looking at a particular compound and see if there was something (a linker) present. Thus, there is no lack of definiteness based on this term in the claims.

The Examiner also states that the characterization of the linker as having a length of 4-7 carbons is unclear. The linker extends from one binding moiety to the other. The specification states that "in general the linker will be 1 to 9 carbon atoms in length" (Page 4, lines 16-17) and provides examples of dimers where the linker is 4 to 12 carbons in length (Table 1). The specification further refers on Page 5 to the linkers of differing lengths depicted in Fig. 2. Thus, a person skilled in art, having read the specification would understand that the phrase "a length of 4 to 7 carbon atoms" is indicative of a chain of 4 to 7 carbon atoms that extends between the two ansamycin antibiotics. There may or may not be substitutions on this chain, or unsaturation (for example a double bond) as indicated on Page 4, line lines 17-19, and Fig. 1.

#### B. The Term "Bind"



The Examiner asserts that the scope of the compounds claimed is indeterminate because the term "bind" is indefinite. Applicants submit that this basis for rejection is in error, and the Board of Appeal agreed in the related case, and indeed stated that "we cannot understand the examiner's concern in this regard ... because 'bind' is an art recognized term, and it is unclear if 'bind' is unacceptable, what term would be acceptable to the examiner." (Page 7)

In support of his position, the Examiner argues that "there is no way of knowing whether a given compound would bind," and that "binding is a process which cannot be observed, merely inferred, which is unreliable." Applicants point out, however, that the art of record in this case establishes that persons skilled in the art have been observing the interaction between ansamycin antibiotics such as geldanamycin with hsp90 for some time, and calling this interaction "binding." For example, the Stebbins reference (of record, attached in the Exhibit Appendix) refers to binding of geldanamycin to hsp90 as an observed phenomenon, and then determines the crystal structure of hsp90 and the location of the binding pocket.<sup>2</sup> Thus, both the meaning of binding, and the specific pocket referred to are clear.

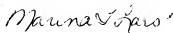
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<sup>2</sup> It is noted that the crystallographic observation of the geldanamycin - hsp90 complex contradicts the Examiner's assertion that the binding cannot be observed. However, such observation is not necessary for the claims to be definite.

Conclusion

For these reasons, Applicants submit that the rejections under 35 USC § 112, first and second paragraphs are in error and should be reversed.

Respectfully submitted,

A handwritten signature in cursive script, appearing to read "Marina T. Larson".

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## Claims Appendix

35. A chemical compound comprising first and second hsp-binding moieties which bind to the pocket of hsp90 with which ansamycin antibiotics bind leading to degradation in proteasomes of a subset of proteins requiring hsp90 for conformational maturation, said binding moieties being connected to one another by a linker, wherein the first and second hsp-binding moieties each retain the ability in the chemical compound to bind to the pocket of hsp90 and lead to degradation in proteasomes of a subset of proteins requiring hsp90 for conformational maturation.

36. The chemical compound of claim 35, wherein at least one of the hsp-binding moieties is geldanamycin, and the linker is connected to the 17-carbon of the geldanamycin.

37. The chemical compound of claim 36, wherein the linker has a length of 4 to 7 carbon atoms.

38. The chemical compound of claim 36, wherein the linker is a substituted carbon chain.

39. The chemical compound of claim 38, wherein the linker is a substituted carbon chain incorporating a secondary or tertiary amine.

40. The chemical compound of claim 39, wherein the linker is an N-methyl amino linker.

41. The chemical compound of claim 35, wherein the linker is a substituted carbon chain.

42. The chemical compound of claim 41, wherein the linker is a substituted carbon chain incorporating a secondary or tertiary amine.

43. The chemical compound of claim 42, wherein the linker is an N-methyl amino linker.

44. A method for destruction of cells expressing a HER-family tyrosine kinase, comprising administering to the cells a chemical compound comprising first and second hsp-binding moieties which bind to the pocket of hsp90 with which ansamycin antibiotics bind leading to degradation in proteasomes of a subset of proteins requiring hsp90 for conformational maturation, said binding moieties being connected to one another by a linker, wherein the first and second hsp-binding moieties each retain the ability in the chemical compound to bind to the

pocket of hsp90 and lead to degradation in proteasomes of a subset of proteins requiring hsp90 for conformational maturation.

45. The method of claim 44, wherein at least one of the hsp-binding moieties is geldanamycin, and the linker is connected to the 17-carbon of the geldanamycin .

46. The method of claim 45, wherein the linker has a length of 4 to 7 carbon atoms.

47. The method of claim 46, wherein the linker is a substituted carbon chain.

48. The method of claim 47, wherein the linker is a substituted carbon chain incorporating a secondary or tertiary amine.

49. The method of claim 38, wherein the linker is an N-methyl amino linker.

50. The method of claim 44, wherein the linker is a substituted carbon chain.

51. The method of claim 50, wherein the linker is a substituted carbon chain incorporating a secondary or tertiary amine.

52. The method of claim 51, wherein the linker is an N-methyl amino linker.

53. A method for treating cancer in a patient suffering from cancer, comprising administering to the patient a therapeutic composition comprising a chemical compound comprising first and second hsp-binding moieties which bind to the pocket of hsp90 with which ansamycin antibiotics bind leading to degradation in proteasomes of a subset of proteins requiring hsp90 for conformational maturation, said binding moieties being connected to one another by a linker, wherein the first and second hsp-binding moieties each retain the ability in the chemical compound to bind to the pocket of hsp90 and lead to degradation in proteasomes of a subset of proteins requiring hsp90 for conformational maturation.

54. The method of claim 53, wherein the cancer is an HER-positive cancer.

55. The method of claim 54, wherein at least one of the hsp-binding moieties is geldanamycin, and the linker is connected to the 17-carbon of the geldanamycin .

56. The method of claim 55, wherein the linker has a length of 4 to 7 carbon atoms.

57. The method of claim 56, wherein the linker is a substituted carbon chain.

58. The method of claim 57, wherein the linker is a substituted carbon chain incorporating a secondary or tertiary amine.
59. The method of claim 58, wherein the linker is an N-methyl amino linker.
60. The method of claim 54, wherein the linker is a substituted carbon chain.
61. The method of claim 60, wherein the linker is a substituted carbon chain incorporating a secondary or tertiary amine.
62. The method of claim 61, wherein the linker is an N-methyl amino linker.
63. The method of claim 53, wherein the cancer is breast cancer.
64. The method of claim 53, wherein the cancer is ovarian cancer.
65. The method of claim 53, wherein the cancer is pancreatic cancer.
66. The method of claim 53, wherein the cancer is gastric cancer.

## Evidence Appendix

Yang et al. (Exhibit A)  
Okabe et al. (Exhibit B)  
Kelland *et al* (Exhibit C)  
Burger *et al* (Exhibit D)  
Banerji et al. (Exhibit E)  
Solit et al. (Exhibit F)  
Nguyen et al. (Exhibit G)  
Nguyen et al. (Exhibit H)  
Stebbins Article

# Disruption of the EF-2 Kinase/Hsp90 Protein Complex: A Possible Mechanism to Inhibit Glioblastoma by Geldanamycin<sup>1</sup>

④ Glioma

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## ABSTRACT

Glioblastoma multiforme is the most treatment-resistant brain tumor. Elongation factor-2 (EF-2) kinase (calmodulin kinase II) is a unique protein kinase that is overexpressed in glioma cell lines and in human surgical specimens. Several mitogens activate this kinase and inhibitors block mitogen activation and produce cell death. Geldanamycin (GA) is a benzoquinone ansamycin antibiotic that disrupts Hsp90-protein interactions. Because EF-2 kinase is chaperoned by Hsp90, we investigated the effects of GA on the viability of glioma cells, the expression of EF-2 kinase protein, and the interaction between Hsp90 and EF-2 kinase. GA was a potent inhibitor of the elongogenicity of four glioma cells lines with IC<sub>50</sub>s ranging from 1 to 3 nM. 17-allylamino-17-demethoxygeldanamycin (17-AAG), a less toxic and less potent derivative of GA, inhibited the elongogenicity of glioma cells with IC<sub>50</sub> values of 13 nM in C6 cells and 35 nM in T98G cells. Treatment of cell lines for 24–48 h of GA or 17-AAG disrupted EF-2-kinase/Hsp90 interactions as measured by immunoprecipitation, resulting in a decreased amount of recoverable kinase in cell lysates. The ability of GA to inhibit the growth of glioma cells was abrogated by overexpressing EF-2 kinase. In addition, 17-AAG significantly inhibited the growth of a glioma xenograft in nude mice. These studies demonstrate for the first time the activity of GAs against human gliomas *in vitro* and *in vivo* and suggest that destruction of EF-2 kinase may be an important cytotoxic mechanism of this unique class of drug.

## INTRODUCTION

Glioblastoma multiforme is a highly resistant, lethal malignancy of the central nervous system that represents an increasingly important cause of death from cancer in adults and children (1). Current therapy with surgery, radiation, and chemotherapy rarely, if ever, cures the disease and infrequently prolongs life for more than 1 year (2). Therefore, our laboratory has been investigating new signal transduction proteins as potential targets for drug development.

Activation of tyrosine kinases through receptor occupation or mutation initiates several signal transduction pathways that culminate in cell division. The epidermal growth factor receptor is frequently overexpressed or mutated in human glioblastoma (3–5). Activation of phospholipase C by the epidermal growth factor receptor produces two second messengers, diacylglycerol and inositol-1,4,5-trisphosphate. Whereas the former can participate in mitogen-activated protein kinase signaling, the latter activates calmodulin-dependent pathways through the release of intracellular calcium (6–9).

We previously found that calmodulin-dependent phosphorylation of eEF-2<sup>3</sup> was markedly increased in glioblastoma because of the increased activity of calmodulin-dependent protein kinase 3 (10), also

known as EF-2 kinase (11–13). EF-2 kinase phosphorylates eEF-2 in response to elevation in intracellular calcium, which leads to the inactivation of this translation factor (14, 15). Additional studies defined EF-2 kinase as a proliferation-dependent and mitogen-activated enzyme in a variety of normal and malignant cell types (16, 17).

The cloning and sequencing of EF-2 kinase led to the realization that it represented a unique enzyme family (13). Except for the ATP binding site, EF-2 kinases from several different species exhibit no sequence homology to any Ca<sup>2+</sup>/calmodulin-dependent protein kinase, or to any member of the eukaryotic protein kinase superfamily. However, EF-2 kinase does have homology to the catalytic domain of myosin heavy chain kinase A (MHCK A) from *Dicystostelium*. The unique structural features of EF-2 kinase, together with its increased activity in malignancy and cell-cycle dependency, makes it a novel target for anticancer therapies.

In fact, several lines of evidence suggest that inhibiting the activity of EF-2 kinase can kill cancer cells. For example, Parmer *et al.* (18), demonstrated that rottlerin, an EF-2 kinase inhibitor, blocked glioma cells at the G<sub>1</sub>-S phase interface and killed a variety of cell lines at concentrations (1–10 nM) that were consistent with IC<sub>50</sub>s for enzyme inhibition (18). Antisense RNA to calmodulin (19) and EF-2 kinase<sup>4</sup> markedly decreased the clonogenicity of rat and human glioma cell lines. Therefore, identification of new drugs that block the function of EF-2 kinase may lead to new types of anticancer drugs.

During the purification of EF-2 kinase (20), we found the enzyme to be tightly associated with Hsp90 as previously demonstrated by Palmquist *et al.* (21). Hsp90 is a protein chaperone responsible for maintaining proper protein folding and stability (22). Recently, a new class of drug, the GAs was found to disrupt Hsp90/protein interactions (23). These benzoquinone ansamycin antibiotics were shown to have antitumor effects in cell culture and experimental animals (24). Recently, 17-AAG, a less toxic and less potent derivative of GA, has entered Phase I clinical testing (25–27).

Because the activity of EF-2 kinase is markedly increased in human glioblastoma and is chaperoned by Hsp90, we examined the effects of the GAs on this lethal malignancy of the central nervous system.

## MATERIALS AND METHODS

**Cell Culture.** The C6 *N*-nitrosomethylurea-induced rat glioma line and the human cell lines T98G (glioblastoma), Daoy (medulloblastoma), and SKNSH (neuroblastoma) were obtained from The American Type Culture Collection. TJY1-R and TJY2-D cell lines were derived from T98G cells by stable transfection with either antisense or sense cDNA of EF-2 kinase. These two cell lines expressed either low or high amounts of EF-2-K protein, respectively, relative to the parental line. T98G and Daoy cells were grown in a 1:10 DMEM:Ham's F-10 media, supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 mg/ml streptomycin. TJY1-R and TJY2-D cell lines were maintained in the same medium as T98G, but were supplemented with 200 µg/ml G418. C6 and SKNSH cells were cultured in supplemented DMEM. Cell cultures were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Cells in log phase were grown in 100-mm tissue culture plates and treated with GA or 17-AAG as indicated in the figure legend.

<sup>4</sup> S. Hsu, J. Yang, and W. N. Hait, unpublished observations.

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<sup>3</sup> The abbreviations used are: eEF-2, elongation factor 2; EF-2 kinase, elongation factor 2 kinase; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; GA, geldanamycin; Hsp90, heat shock protein 90; PMSE, phenylmethylsulfonyl fluorid.

Drugs: GA and 17-AAG were obtained from Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis of the National Cancer Institute in the form of a lyophilized powder. They were stored in dark, airtight containers at 4°C and reconstituted in DMSO immediately before use.

**Clonogenic Assays.** Cytotoxicity of GA and 17-AAG was determined by clonogenic assay. Four  $\times 10^5$  C6 cells or  $5 \times 10^5$  T98G cells were plated in 60-mm tissue culture dishes and allowed to adhere overnight. The cells were then exposed to various concentrations of drug ranging from 0.05–100 nM (control cultures were treated with 0.01% DMSO) and incubated for 24–48 h at 37°C. The medium was then removed and replaced with fresh medium free of drug. After 7–10 days of growth, colonies were fixed and stained with 0.5% methylene blue. Colonies with diameters of 2 mm and larger were counted using an electronic counting pencil.

**Preparation of Cell Homogenates for Detection of Cellular EF-2 Kinase.** Cell monolayers were washed twice in PBS (pH 7.4), scraped into 15-ml centrifuge tubes in 5 ml of PBS, and then centrifuged at 1,000  $\times$  g for 5 min. Cell pellets were homogenized with 15 strokes in ice-cold HOMO buffer (25 mM HEPES (pH 7.4), 100 mM sodium chloride, 20 mM sodium PP<sub>i</sub>, 2 mM EDTA, 0.1 mM PMSE, 10  $\mu$ M leupeptin, 2  $\mu$ M pepstatin A, and 0.1 mM sodium orthovanadate), using a Polytron homogenizer. The homogenates were then centrifuged at 15,000  $\times$  g for 30 min at 4°C. The protein concentration of the supernatants was determined according to the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Twenty  $\mu$ g of protein from each sample were used for EF-2 kinase detection. Sample volumes were adjusted with HOMO buffer. Samples were boiled with 3X Laemmli buffer [190 mM Tris (pH 6.8), 6% SDS, 30% glycerol, 15% 2-mercaptoethanol, and 0.005% bromophenol blue dye] for 5 min before resolution by 7.5% SDS-PAGE.

**Immunoprecipitations.** Cell monolayers were washed twice with PBS and lysed with NET lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP40, 1.0 mM EDTA, 1 mM PMSE, and 1% aprotinin] at 4°C for 30 min. Cells were scraped into 1.5-ml microfuge tubes and incubated at 4°C with rotation for 30 min, passed through a 25-gauge needle, and then centrifuged at 15,000  $\times$  g at 4°C for 30 min. The protein concentration of the supernatants was determined using the Bio-Rad protein assay kit.

**Immunoprecipitations** were performed using 2 mg (C6 cells) or 1 mg (T98G cells) of protein from total cell lysates as starting material. Reaction volumes were equalized using NET buffer. For Hsp90 precipitation, 4  $\mu$ g of anti-Hsp90 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were added to each sample. For EF-2 kinase precipitation, 4  $\mu$ l of anti-EF-2 kinase rabbit serum (kindly provided by Dr. A. C. Nairn of Rockefeller University, New York, NY) was used. Immunoprecipitation of samples with preimmune sera was used as a control. After 1–4 h of rotating incubation at 4°C, 80  $\mu$ l of Protein A-Sepharose CL-4B (50% NET buffer; Amersham Pharmacia Biotech, Piscataway, NJ) were added. Tubes were incubated overnight at 4°C with rotation. At the end of the incubation, samples were centrifuged at 15,000  $\times$  g at 4°C for 2 min, and the supernatant was discarded. Beads were washed twice with NET buffer and twice with PBS containing 1% aprotinin and 1 mM PMSE. After the final wash, 30  $\mu$ l of Laemmli buffer were added, and the samples were boiled for 5 min. After a 3-min spin at 15,000  $\times$  g, supernatants were loaded onto a 8% SDS-PAGE gel.

**Western Blot Analysis.** Proteins resolved on SDS-PAGE were transferred to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech). Membranes were first blocked with 10% milk in PBS-T (PBS-0.1% Tween) and then incubated with anti-EF-2 kinase rabbit antiserum in 10% milk/PBS-T for EF-2 kinase detection. For detection of Hsp90, the anti-Hsp90 rabbit antiserum was used. An anti- $\beta$ -actin monoclonal mouse ascites (Sigma Chemical Co., St. Louis, MO) was used for detection of  $\beta$ -actin. After a 2-h incubation with the primary antibodies, membranes were washed in PBS-T, incubated with horseradish peroxidase conjugated secondary antibodies (Amersham-Life Science), and washed again with PBS-T.  $M_r$  standards (Bio-Rad Laboratories, Richmond, CA) and authentic EF-2 kinase were used to determine the position of the proteins of interest. Proteins were visualized using Amersham-Pharmacia ECL detection reagent. Blots were scanned by UMAX Magic Scan 4.3 program, and the intensity of protein bands was quantified by Molecular Analyst software.

**Generation of F<sup>-</sup> Cell Lines Expressing Different Levels of EF-2 Kinase.** pSTAR was originally constructed to mediate tetracycline-induced gene expression in mammalian cells (28). However, this vector conferred constitutive expression of target genes in most stable clones that were cytomegalovirus and SV40, in this vector system. A full-length human EF-2 cDNA was inserted into the multiple cloning site region of pSTAR in both antisense (AS) and sense (S) orientations. Constructs expressing AS (pYH1) and sense (pYH2) EF-2 kinase mRNA were introduced into T98G human glioma cells by liposome-mediated transfection (Life Technologies, Inc., Rockville, MD). Transfectants were selected in 500  $\mu$ g/ml G418. G418-resistant colonies were expanded and maintained in 200  $\mu$ g/ml G418. Cytosolic extracts of each clone were collected by homogenization and centrifugation and were analyzed for EF-2 kinase expression by Western blot. The TJY1-R clone was found to express low amounts of EF-2 kinase protein. The TJY2-D clone expressed high levels of sense EF-2 kinase mRNA and protein compared with that of TJY1-R.

**Treatment of Glioma Xenografts with 17-AAG.** C6 cells grown at logarithmic phase were removed from culture flasks by trypsinization, then washed and resuspended in PBS at density of  $1.6 \times 10^7$  cells/ml. Cells ( $1.6 \times 10^7$  cells) were injected into the flank of each of 16 Swiss nude (*nu/nu*) mice (male, 5 weeks old; Taconic, Germantown, NY). The s.c. grown tumors were measured daily with a Vernier caliper. The length (L) and width (W, in mm) of the tumors were taken and used to calculate the volume (V), using the formula:  $V = (W^2 \times L) / 2$ . On the 12th day after inoculation, when the median tumor volume reached 60 mm<sup>3</sup>, the mice were paired by the similarity of the tumor volumes. Within each pair, mice were randomly assigned to either 17-AAG or vehicle. Animals received i.p. injections of 17-AAG (80 mg/kg) or vehicle on days 2, 3, 4, 5, and 8, 9, 10, 11, and 12.

**Statistical Analysis.** The two-sample *t* test was used to compare tumor volumes between the two treatment groups at treatment day 1. Log(tumor volume) on day 5 to day 22 were analyzed using the repeated-measurement model with treatment and day as factors and the first order autoregressive correlation structure (29).

## RESULTS

**Effect of GA on Cell Growth and Viability.** We investigated the effects of a 48-h incubation with GA and 17-AAG on the clonogenicity of human T98G (glioblastoma), Daoy (medulloblastoma) and SKNSH (neuroblastoma) cells. GA inhibited T98G, Daoy, and SKNSH cell viability with IC<sub>50</sub> values of 3 nM, 1 nM, and 1 nM, respectively (Fig. 1A; Table 1). GA also inhibited the clonogenicity of the C6 rat glioma line (IC<sub>50</sub>, 1.5 nM).

17-AAG also decreased the clonogenicity of glioma cells, but was ~10-fold less potent than GA against the same cell lines. IC<sub>50</sub> value for C6 cells was 13 nM and for T98G cells was 35 nM (Fig. 1B; Table 1).

For comparison, we also performed clonogenic assays of C6 cells treated with BCNU, a standard chemotherapeutic drug used to treat glioblastoma. Fig. 1C and Table 1 show that the IC<sub>50</sub> of BCNU against C6 cells was 5  $\mu$ M, ~3000-fold greater than the IC<sub>50</sub> value of GA against the same cell line.

**Effect of GA and 17-AAG on EF-2 Kinase Protein Content.** To analyze the role of EF-2 kinase in the cytotoxicity of GAs, we tested whether or not GA or 17-AAG affected EF-2 kinase content in glioma cells. T98G cells were incubated with 0–40 nM GA for 24 h (Fig. 2A, left panel). Cell homogenates were collected and EF-2 kinase measured by Western blot. Fig. 2 demonstrates that there was a >50% decrease in EF-2 kinase at a concentration of 10 nM and a >80% decrease after treatment with 20 nM GA. We chose the 24-h incubation time to avoid problems in interpretation of data obtained using a dying population of cells (Fig. 1). Similar results were observed in C6 cells treated with GA for 24 h (Fig. 2B); EF-2 kinase was reduced by 70% after treatment with 1–10 nM GA. 17-AAG (20–40 nM) decreased EF-2 kinase by 40–50% (Fig. 2A, right panel).



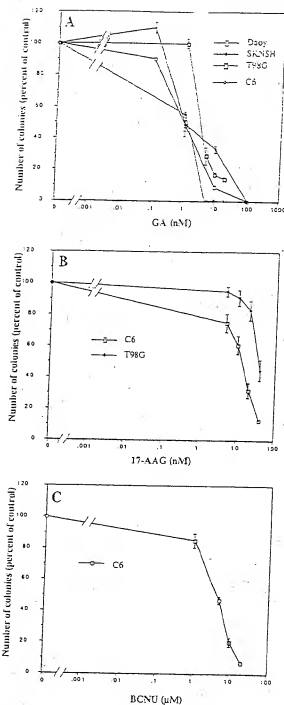


Fig. 1. Effect of GA, 17-AAG, and BCNU on the clonogenic survival of glioma and other cell lines of neurogenic origin. Murine glioma (C6), human glioblastoma (T98G), human neuroblastoma (SKNSH), and human medulloblastoma (Dasy) were cultured as described in "Materials and Methods." Cell lines were exposed for 48 h in varying concentrations of GA (A), 17-AAG (B), and BCNU (C). Cells were then cultured in drug-free medium and allowed to grow for another 7–10 days. Colonies were stained with methylene blue and counted with an electron counting pencil. Each point represents the mean of three determinations from one of three similar experiments; bars = SE. Controls (vehicle-treated cells) usually contained 100–300 colonies.

**Effect of GA and 17-AAG on Hsp90/EF-2 Kinase Interactions.** We next carried out immunoprecipitation experiments to investigate the effects of GA and 17-AAG on Hsp90/EF-2 kinase protein interactions. T98G (Fig. 3A) and C6 cells (Fig. 3B) were treated with 10 nM GA, 40 nM 17-AAG, or vehicle (0.1% DMSO) for 24 h (the optimal conditions for reducing the cellular content of EF-2 kinase as shown above). Cell lysates were incubated with anti-Hsp90 rabbit

antiserum or preimmune serum and protein-A beads to immunoprecipitate Hsp90. After Western transfer, the membranes were first blotted with anti-EF-2 kinase polyclonal antibody and then were stripped and reblotted with anti-Hsp 90 polyclonal antibody. Fig. 3, A and B, demonstrate that approximately equal amounts of Hsp90 (bottom panels) were precipitated under each condition. EF-2 kinase (upper panels) was coprecipitated with Hsp90 in vehicle-treated cells (Lane 1) but not with preimmune serum (data not shown), which confirmed the association of the kinase with Hsp90 in T98G and C6 cells. Treatment with GA (Lane 2) or 17-AAG (Lane 3) significantly reduced the amount of EF-2 kinase that was coprecipitated with Hsp90 in both cell lines.

To exclude the possibility that the apparent decrease of Hsp90 association with EF-2 kinase was merely the result of decreased overall EF-2 kinase protein, we investigated the effect of shorter incubation times with higher concentrations of drug. In these experiments, cells were incubated with 3  $\mu$ M GA (Lane 4) or 6  $\mu$ M 17-AAG (Lane 5) for 3 h. Fig. 3C demonstrates that the amount of EF-2 kinase in T98G or C6 cells was not significantly changed under these conditions. The amount of EF-2 kinase that was coimmunoprecipitated with Hsp90 under these conditions was markedly decreased (Fig. 3, A and B, Lanes 4 and 5).

We next determined whether antibodies to EF-2 kinase could immunoprecipitate Hsp90 and whether GA disrupted the interaction. Immunoprecipitation was performed using an anti-EF-2 kinase anti-

Table 1. Effect of GAs on the clonogenicity of glioma and other cell lines of neurogenic origin

Cell lines, grown in tissue culture, were treated with varying concentrations of drugs or vehicles, and clonogenic survival was measured as described in "Materials and Methods." Each value represents the mean of at least two experiments having less than 10% variation between them.

Cell line	GA IC <sub>50</sub> (nM)	17-AAG IC <sub>50</sub> (nM)	BCNU IC <sub>50</sub>
C6	2 $\pm$ 0.1	13 $\pm$ 1.8	5 $\pm$ 0.2
T98G	3 $\pm$ 0.2	35 $\pm$ 4.0	
SKNSH	1 $\pm$ 0.1		
Dasy	1 $\pm$ 0.1		
TJY1-R	0.4 $\pm$ 0.04		
TJY2-D	2 $\pm$ 0.1		

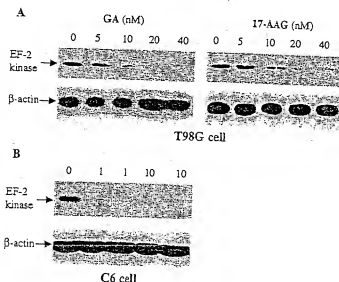


Fig. 2. Effect of GAs on the content of EF-2 kinase in glioblastoma cell lines. T98G (A) and C6 cells (B) were grown in culture as described in "Materials and Methods." Following a 24-h incubation with drug or appropriate vehicle, cell homogenates containing 20  $\mu$ g of total protein were assayed for the content of elongation factor 2 kinase and  $\beta$ -actin by Western blot analysis. The figure represents the results of one of three similar experiments.

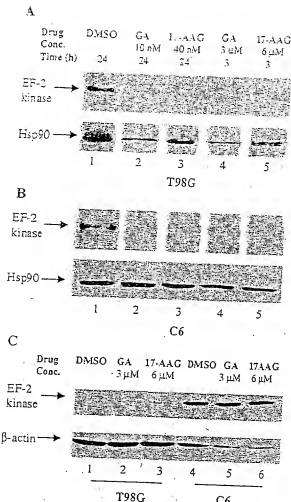


Fig. 3. Immunoprecipitation of EF-2 kinase by antibodies against Hsp90 in cell lysates treated with GA. T98G (A) and C6 (B) cells were treated with 10 nM GA (Lane 2), 40 nM 17-AAG (Lane 3), or vehicle (0.1% DMSO, Lane 1) for 24 h. Cells were also treated with 3 μM GA (Lane 4) or 6 μM 17-AAG (Lane 5) for 3 h. Cell lysates were incubated with anti-Hsp90 rabbit antiserum and protein-A beads to immunoprecipitate Hsp90. After Western transfer, the membranes were first probed with anti-EF-2 kinase polyclonal antibody. C, Western blot analysis of EF-2 kinase in T98G and C6 cells treated with GA or 17-AAG in high concentration and shorter incubation time. Cells were incubated with vehicle (Lanes 1 and 4), 3 μM GA (Lanes 2 and 5), or 6 μM 17-AAG (Lanes 3 and 6) for 3 h. Each blot is representative of three similar experiments.

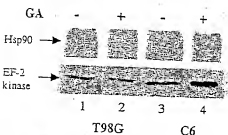


Fig. 4. Immunoprecipitation of Hsp90 by antibodies against EF-2 kinase in cell lysates treated with GA. T98G (A) and C6 (B) cells were treated with either 3 μM GA or vehicle (0.1% DMSO) for 3 h. Cell lysates were incubated with anti-EF-2 kinase rabbit antiserum and protein-A beads as described in "Materials and Methods." After Western transfer, the membrane was first probed with anti-Hsp90 polyclonal antibody, then was stripped and reprobed with anti-EF-2 kinase polyclonal antibody. These data are representative of two similar experiments.

body and cell lysates from T98G and C6 were treated with either vehicle or 3 μM GA for 3 h (Fig. 4). Similar amounts of EF-2 kinase were recovered under each condition (lower panel). Hsp90 was co-precipitated with EF-2 kinase in vehicle controls (Lanes 1 and 3) but

was absent in the immune-complex from GA-treated cell lysates (Lanes 2 and 4).

**Effect of Overexpression of EF-2 Kinase on GA Activity.** We next determined whether the effect of GA on cytotoxicity could be abrogated by overexpressing EF-2 kinase. In these experiments, we compared the sensitivity to GA in two cell lines that express different levels of EF-2 kinase. TJY1-R and TJY2-D cell lines were isolated after stable transfection of T98G cells with either antisense or sense EF-2 kinase expression vectors. TJY1-R was selected based on low kinase expression after transfection with antisense RNA; TJY2-D was selected as a clone expressing high EF-2 kinase after transfection with sense RNA. The Western blot in Fig. 5A shows that TJY2-D cells express ~6-fold more EF-2 kinase protein as compared with that of TJY1-R cells.

Clonogenic assays were performed on TJY1-R and TJY2-D cells treated with different concentrations of GA for 24 h. Overall, we noted a decreased viability of clones transfected with EF-2 kinase antisense. Of the several clones identified, TJY1-R was the most robust based on morphology and a doubling time that was consistent with the sense transfectants (data not shown). Fig. 5B and Table 1 demonstrate that cells that overexpress EF-2 kinase were 5-fold less sensitive to the cytotoxic effects of GA ( $IC_{50}$ , 2 nM) than isogenic cells with less EF-2 kinase content ( $IC_{50}$ , 0.4 nM).

**Effect of 17-AAG on Glioma Growth in Mice.** To test whether GAs remained active against glioma cell lines *in vivo*, we evaluated the effect of 17-AAG on the growth of C6 glioma cells in nude mice. C6 cells were implanted in sixteen mice in the dorsal flank; tumors were allowed to form for 12 days and reached 60 mm<sup>3</sup> in size before

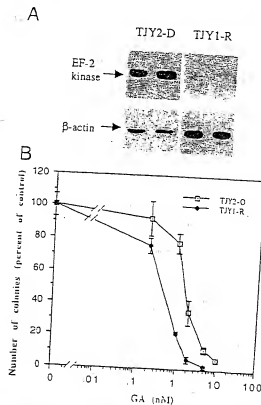


Fig. 5. GA sensitivity of T98G cell derivatives expressing different levels of EF-2 kinase. A, expression of EF-2 kinase in T98 cells transfected with EF-2 kinase sense (TJY2-D) or antisense (TJY1-R) expression vectors. EF-2 kinase and β-actin were detected by Western blot analysis. B, effect of GA on the clonogenic survival of TJY2-D and TJY1-R cell lines. Drug sensitivity was determined by exposing cells to 0–10 nM GA for 24 h, by washing and allowing the cells to grow for another 7–10 days, and by staining with methylene blue. Colonies were then enumerated using an electronic counting pencil as described in "Materials and Methods." Each point represents the mean from a representative of two similar experiments; bars,  $\pm$  SE.

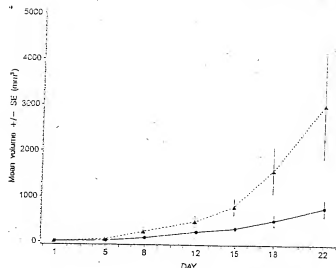


Fig. 6. Effect of 17-AAG on the growth of C6 glioma xenografts. C6 cells ( $1.6 \times 10^6$ /cell/mouse) were implanted into the flank of 16 Swiss nude (*nu/nu*) mice. On the 12th day after inoculation (day 1 in Fig. 6), the mice were paired in two groups and were randomly assigned to treatment. 17-AAG (80 mg/kg) or vehicle were injected via the intraperitoneal route on days 2, 3, 4, 5, 8, 9, 10, 11, and 12. Each point represents the mean volume of the eight animals in each group; bars = SE. The differences in tumor volumes between the two treatment groups were statistically significant ( $P \leq 0.017$ ) at all time points from day 5 to day 22 [least-square mean of  $\log(\text{tumor volume})$ ,  $0.91 \pm 0.33$  (mean  $\pm$  SE)].

treatment began. Fig. 6 demonstrates the ability of 17-AAG to significantly inhibit the growth of C6 glioma. The mean tumor volumes of the two treatment groups were not statistically different before drug treatment (day 1;  $P > 0.752$ ). The differences in tumor volumes between the two treatment groups were statistically significant ( $P \leq 0.017$ ) at all time points from day 5 to day 22 [least-square means of  $\log(\text{tumor volume})$ ,  $0.91 \pm 0.33$  (mean  $\pm$  SE)]. No significant weight loss, changes in activity or deaths were observed during the period of drug treatment.

## DISCUSSION

The need for new treatments for glioblastoma is highlighted by its devastation to both children and adults. Despite the recent introduction of an oral alkylating agent, temozolomide (30–32), very little has been added to prospects for increasing overall survival in patients with this disease.

Disruption of Hsp90 chaperoning represents a novel approach to developing new anticancer therapies. Numerous proteins induced in the growth of malignant cells associate with Hsp90 (22, 33). Interference with this interaction targets the nonassociated protein for degradation (34–38). Therefore, it may be possible to target proteins important for the growth of malignant cells by disrupting their association with Hsp90.

EF-2 kinase is an attractive target for the treatment of high-grade astrocytomas and perhaps other malignancies. The markedly increased activity of this enzyme in glioblastoma multiforme (17) and the cytotoxicity of specific and nonspecific inhibitors for this kinase support this concept (18). Because EF-2 kinase is chaperoned by Hsp90 (20, 21), we examined the effects of GAs on glioma and other malignant cell lines of neurogenic origin and found these drugs to be potent cytotoxic agents that disrupt Hsp90/EF-2 kinase interactions.

GA is a potent inhibitor of the clonogenicity of several cell lines including T98G and C6 glioblastoma, Daoy medulloblastoma, and SKNSH neuroblastoma (Fig. 1). Table I lists the  $IC_{50}$  values obtained after a 48-h exposure to GA that ranged from 1 to 3 nM. For C6 cells, GA is 2500 times more potent than BCNU, a standard chemotherapy

for glioblastoma (39–42). 17-AAG, a less potent inhibitor of Hsp90/protein interactions (25, 26) compared with GA, is also a less potent inhibitor of clonogenicity in T98G and C6 cells, with  $IC_{50}$  values ranging from 13 to 35 nM (Fig. 1, Table I). It is nevertheless a potent compound when compared with BCNU.

To determine whether or not the cytotoxicity of GAs was attributable, at least in part, to inhibition of EF-2 kinase activity through disruption of Hsp90 chaperoning, we carried out a series of experiments to measure both the association and depletion of the enzyme. These studies revealed a good correlation between the concentration of drug required to disrupt Hsp90/EF-2 kinase interactions and effects on clonogenic survival. For example, after a 24 h incubation of T98G cells with 10 nM GA there is a  $>50\%$  decrease in EF-2 kinase (Fig. 24), which correlates with the  $IC_{50}$  of 3 nM obtained by clonogenic assay after exposing the cells to drug for 48 h (Fig. 1, Table I). We chose the shorter incubation period to evaluate the effect of drug exposure on kinase degradation to avoid the pitfalls of studying the effects of drug in a population in which one-half the cells were dead or dying. Similarly, we found a 70% decrease in EF-2 kinase in C6 cells exposed for 24 h to 10 nM GA, which correlates with the  $IC_{50}$  value of 2 nM obtained by clonogenic assay after exposing the cells to drug for 48 h. The  $IC_{50}$ s in clonogenic assays for 17-AAG were 5- to 10-fold greater than those of GA, which correlates to the decreased potency of this derivative in producing loss of the kinase (Fig. 24).

Interference with the chaperoning function of Hsp90 is known to be detrimental to associated kinases. GA disrupts the binding of EF-2 kinase to Hsp90 as measured by coimmunoprecipitation (Fig. 3 and 4). The disruption of this interaction is associated with decreased EF-2 kinase protein content (Fig. 2). Schulte *et al.*, demonstrated that the serine/threonine kinase, raf-1, is rapidly destabilized on addition of GA, which disrupts the Raf-1/Hsp90 molecular complex (38). The disappearance of EF-2 kinase in glioma cells treated with GA is likely caused by this mechanism. These data are consistent with a proposed mechanism by which GAs disrupt the chaperoning of critical cellular proteins leading to targeted degradation (38, 43, 44). Further insight may be gained by investigating the mechanism by which the EF-2 kinase protein is degraded. Published reports (45–47) point toward the role of GA in targeting proteins for proteasome degradation.

GAs are likely to affect numerous intracellular targets that are critical for cell viability. In addition to raf-1 (26, 48–50), both GA and 17-AAG deplete cells of the transmembrane protein tyrosine kinase p185<sup>HER-2</sup>, which has been implicated in oncogenic transformation (45, 51). Treatment of lung cancer cell lines expressing p185 with paclitaxel and 17-AAG showed synergistic activity (52). 17-AAG also destabilizes mutant forms of p53 (47). Therefore, to understand the importance of EF-2 kinase among these other targets, we transfected parental T98G cells with either sense or antisense EF-2 kinase expression vectors. As expected, overexpression of antisense mRNA and subsequent decrease in EF-2 kinase (Fig. 5A) reduced the viability and subsequent recovery of transfected clones (53). Nonetheless, we were able to study drug sensitivity by choosing our most robust antisense clone for these experiments and compared this with a sense clone obtained during the same round of transfections. We then chose a shorter drug exposure time (24 h) to measure effects on clonogenicity and repeatedly found the antisense clone to be more sensitive to GA (Fig. 5B). This result is consistent with EF-2 kinase being an important target for the cytotoxicity of GAs in glioma cell lines. However, despite the excellent correlation between EF-2-kinase content and drug sensitivity (Fig. 5, A and B), given the difficulty in recovering viable antisense clones, we cannot rule out the

possibility that these are indirect effects resulting from clonal selection.

Numerous drugs kill cancer cells in tissue culture but far fewer retain activity in animal xenografts. We studied 17-AAG against xenografted C6 glioma because it is a less toxic compound that has already entered clinical testing (26, 27). 17-AAG significantly inhibits the growth of established gliomas in animals. Treatment that is started after 12 days of growth significantly inhibits the growth of the xenografts (Fig. 6). This is particularly promising because the drug is highly lipid-soluble and likely to penetrate the blood-brain barrier.

In summary, our results demonstrate that GAs represent a new class of anticancer drug that have potent activity against glioblastoma and other malignant cell lines of neurogenic origin. The compounds disrupt the interaction of EF-2 kinase with Hsp90, leading to the destruction of this unique protein kinase member of the calmodulin-mediated pathway of signal transduction.

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(4) BCR-ABL - driven  
mouse leukemia

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# IN VIVO ANTITUMOR ACTIVITY OF HERBIMYCIN A, A TYROSINE KINASE INHIBITOR, TARGETED AGAINST BCR/ABL ONCOPROTEIN IN MICE BEARING BCR/ABL-TRANSFECTED CELLS

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**Abstract**—Herbimycin A, a benzoguinoid ansamycin antibiotic, has been shown to reverse the oncogenic phenotype of p60<sup>src</sup> transformed cells because of the inhibition of src protein tyrosine kinase. We previously demonstrated that herbimycin A displayed antitumor activity on the *in vitro* growth of Philadelphia chromosome-positive leukemia cells and BCR/ABL-transfected murine hematopoietic FDC-P2 cells through the inhibition of BCR/ABL protein tyrosine kinase. In this study, the transformed FDC-P2 cells were demonstrated to be tumorigenic in syngeneic DBA/2 mice. The intraperitoneal (i.p.) injection of the transformed tumor cells into DBA/2 mice induced infiltrations of abdominal organs, and then all of the mice died within time periods proportional to the cell numbers of inoculation. In mice that received an i.p. inoculation with greater than  $1 \times 10^5$  cells, *in vivo* administration of herbimycin A by i.p. injection inhibited tumor formation and significantly prolonged survival time, and further, in mice inoculated with  $1 \times 10^4$  cells, herbimycin A completely suppressed the *in vivo* growth of transformant FDC-P2 cells and brought about a complete remission. The present study revealed the *in vivo* efficacy of herbimycin A in mice bearing BCR/ABL-transfected cells.

**Key words:** Herbimycin A, BCR/ABL oncoprotein, protein tyrosine kinase, tyrosine kinase inhibitor, *in vivo* antitumor activity.

## Introduction

The *bcr/abl* oncogene is the product of the Philadelphia chromosome (Ph<sup>1</sup>), resulting from the reciprocal translocation between chromosomes 9 and 22 which are found in Ph<sup>1</sup>-positive leukemias which mainly constitutes more than 90% of chronic myelogenous leukemia (CML) and approximately 20% of adult acute lymphocytic leukemia (ALL) [1-5]. The *bcr/abl* fusion proteins generating from the hybrid *bcr/abl* genes, P210<sup>bcr/abl</sup> and P190<sup>bcr/abl</sup> exhibit the deregulated tyrosine kinase activity of ABL and oncogenic activity *in vitro* and *in vivo* [6-9].

Recent progress in the study of the *bcr/abl* gene and its product in Ph<sup>1</sup>-positive leukemia offers an opportunity for developing several strategies for specific therapy targeted against BCR/ABL [10-14]. Herbimycin A, an inhibitor of protein tyrosine kinase (PTK) that we isolated on the basis of its ability to

cause rat kidney cells transformed by v-src to revert to normal morphology with a loss of PTK activity of the transforming protein of p60<sup>src</sup> [15], was shown to display a relatively selective antitumor activity for transformed cells by oncogenes coding PTK [16]. In fact, we demonstrated that herbimycin A displayed a preferential antitumor activity on the *in vitro* growth of Ph<sup>1</sup>-positive cells by reducing BCR/ABL PTK [12]. In order to determine whether or not herbimycin A may offer a new therapeutic potential for Ph<sup>1</sup>-positive leukemia, we have investigated the antitumor activity of *in vivo* administration of herbimycin A in syngeneic DBA/2 mice inoculated with BCR/ABL oncoprotein-transformed murine FDC-P2 cells. Here, we report the *in vivo* efficacy of herbimycin A on mice bearing BCR/ABL oncoprotein-associated tumors.

## Materials and Methods

### Murine hematopoietic cell lines

The murine IL-3 dependent hematopoietic cell line FDC-P2 [17] was used in this study. As control cells, P815,

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mastocytoma cell line, and P388 lymphoma cell line, were used.

#### Transfection of retroviral vector expressing P210<sup>bcrl/abl</sup> into IL-3-dependent FDC-P2 cells

Five million FDC-P2 cells were cultured by the addition of a mixture of 30  $\mu$ l Lipofectin (Gibco, Berlin, Germany) and 4  $\mu$ g of pGD210 retroviral vectors expressing P210<sup>bcrl/abl</sup> oncoprotein [8] for 16 h in serum-free Opti-MEM (Gibco) in the presence of recombinant murine IL-3 (rmIL-3) which was prepared from COS-1 cells transfected with pSV2neo containing mL-3 cDNA. The cells were selected in a RPMI-1640 medium to which 10% fetal bovine serum (FBS) and 600  $\mu$ g/ml gentamicin (G418 sulfate; Gibco) were added in the presence of rmIL-3 for 10 days, and were then further selected in a medium lacking mL-3 for IL-3-independent growth. In the absence of mL-3, parental or mock pZiPNeoS(X)-transfected FDC-P2 cells died within 72 h, but G418-resistant pGD210<sup>bcrl/abl</sup>-transfected cells were able to grow autonomously in a medium which lacked mL-3. As previously described in detail [12], transformed FDC-P2 cells expressed *bcrl/abl* and *neo* mRNAs and the P210<sup>bcrl/abl</sup> oncoprotein.

#### In vitro growth testing for herbimycin A

The assay of cellular proliferation was carried out by using the colorimetric MTT method. The cells ( $2 \times 10^4$ /well) were seeded in triplicate in 100  $\mu$ l of 10% FBS-added RPMI 1640 with or without herbimycin A, and cultured for 3–5 days in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. After the addition of 10  $\mu$ l of a 5 mg/ml stock solution of MTT (3-(4,5-dimethylthiazol-2,5-diphenyl) tetrazolium bromide) (Sigma Co. Ltd., Munich, FRG) into each well for the last 4 h, formazan crystals were solubilized in 100  $\mu$ l of isopropanol-0.04 N HCl, and then the plates were measured on a MTP-100 microplate reader (Corona Electric, Tokyo, Japan) using a test wavelength of 570 nm with a reference wavelength of 630 nm.

#### Western blotting and immune complex kinase assay of BCR/ABL

Transformant FDC-P2 cells were cultured for 16 h in the presence or absence of 0.2  $\mu$ g/ml of herbimycin A, where cell viabilities of both groups were more than 90%. The cells were lysed in the presence of kinase and phosphatase inhibitors, as previously described by Longo [18]. The lysate was clarified by centrifugation at 4°C, and 20  $\mu$ g of proteins in the supernatant were separated by SDS-PAGE in reducing conditions (100 mM dithiothreitol). Proteins were blotted onto a polyvinylidene difluoride (Millipore) membrane. The BCR/ABL oncoprotein was identified using a monoclonal anti-ABL protein (Ab-3, Oncogene Science, Mineola, NY).

The immune complex kinase assay was carried out by using a modified method of that described by Zuckrock et al. [19, 20]. In brief, the cells ( $1 \times 10^6$ ) were harvested, washed with phosphate-buffered saline (PBS), and then lysed at 4°C by sonication in 50 mM Tris-HCl pH 7.5, containing 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM PMSF. The diluted sample was treated with mouse monoclonal antibody against *abl*. Ab-3 (Oncogene Science), for 1 h at room temperature and was incubated overnight at 4°C. Immune complexes with anti-ABL antibody were precipitated with mouse anti-IgG antibody-coated protein A (Zymed Laboratories, San Francisco, CA), and immunoprecipitates

were then reacted with 5  $\mu$ Ci of ( $\gamma$ -<sup>32</sup>P) ATP for 10 min on ice. Next, immune complexes were analyzed on 7.5% SDS-polyacrylamide gels, followed by autoradiography with an intensifying screen.

#### Animals

Seven-week-old female DBA/2 mice were supplied by Oriental Kobo Co. Ltd (Tokyo, Japan).

#### Examination of tumor cells and tissues

Tissues were processed for histological examination and for Southern blot analysis. High molecular DNAs derived from tissues and tumors were digested with restriction enzymes, electrophoresed through 0.8% agarose gel, and then transferred to nylon membranes. The hybridization with a human 3'-*bcrl* genomic DNA probe (*bcrl*-1; Oncogene Science) was carried out by the method previously described [21].

#### Administration of herbimycin A

Herbimycin A was isolated as described previously [15], and was dissolved in phosphate-buffered saline containing 1% (V/V) of Tween-20. Various concentrations of herbimycin A were then administered by i.p. injections daily from days 2–6 and from day 12 to day 16 after either transformed FDC-P2 cells, P388 cells or P388 cells at cell numbers of  $1 \times 10^4$ – $1 \times 10^6$  were inoculated intraperitoneally into syngeneic DBA/2 mice on the first day.

#### Expression of results

Unless otherwise indicated, mean values  $\pm$  one S.D. for measurements from triplicate culture have been presented. Bars in figures indicate one S.D. The statistical analysis was carried out using a Student's *t*-test, and results were interpreted to be significantly different when  $p < 0.001$ .

## Results

#### In vitro antitumor effect of herbimycin A on BCR/ABL-transformed murine cells

We investigated the *in vitro* effect of herbimycin A against P210<sup>bcrl/abl</sup> oncoprotein-associated autonomous growth of FDC-P2 cells transformed by a transfection of the *bcrl/abl* gene. Herbimycin A showed no significant inhibition of the growth of parental FDC-P2 cells in the presence of the culture supernatant of WEHI cells containing mL-3, but did display an inhibitory effect on the growth of transformant FDC-P2 cells at doses showing no toxicity on murine P381 cells and P388 cells (Fig. 1).

#### Effect of herbimycin A on BCR/ABL oncoprotein and tyrosine kinase

We previously reported that the antitumor activity of herbimycin A on Ph<sup>+</sup>-positive leukemia cells seems to be associated with an inhibition on the activity of BCR/ABL PTK in spite of having no effect on the expression of BCR/ABL oncoprotein [12]. Similarly, the immune complex kinase assay of BCR/ABL revealed that herbimycin A dramatically suppressed

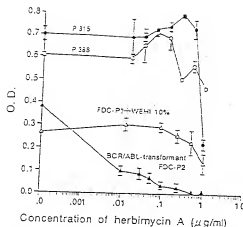


Fig. 1. Effects of herbimycin A on the *in vitro* growth of parental FDC-P2 cells, BCR/ABL-transfected FDC-P2 cells and syngeneic murine hematopoietic malignant cells. Effects of herbimycin A on IL-3-dependent growth of parental FDC-P2 cells in the presence of the culture supernatant obtained from WEHI cells and autonomous growth of transformant FDC-P2 cells by a transfection with BCR/ABL were assayed by MTT proliferation assay after culturing for 72 h. In P815 and P388 cells, MTT proliferation assay was carried out after the 72 h and 5 day cultures, respectively. The growth of P815 cells and P388 cells was not significantly inhibited at low doses of herbimycin A showing an inhibitory effect on the growth of transformant FDC-P2 cells in the 72 h and 5 day cultures. The results of 5 day cultures of P815 and P388 cells are shown.

the autophosphorylation activity of P210<sup>bcrl/abl</sup> tyrosine kinase in BCR/ABL-transformed FDC-P2 cells, while it did not affect the amount of BCR/ABL oncoprotein (Fig. 2).

#### Mice bearing tumor cells transfected with BCR/ABL

Transformant FDC-P2 cells were transplantable by either i.p. or i.v. injection into syngeneic DBA/2 mice, whereas parental FDC-P2 cells were not transplanted. Mice inoculated with transformant FDC-P2 cells by i.p. injections exhibited a gradual increase of abdominal tumors and died within 5–11 weeks post-inoculation. Postmortem examinations revealed that tumor cells formed masses on the abdominal walls and infiltrated mainly into mesenteric and retroperitoneal lymph nodes (Fig. 3(A)). The invasions were observed in the liver, spleen, pancreas and peritoneum (Fig. 3(A)), but were not noted in other organs including the lungs and kidneys. The presence of the *bcr/abl* gene in tumors was confirmed by Southern blot analysis with a human *bcr* probe (Fig. 3(B)).

#### In vivo efficacy of herbimycin A in mice bearing BCR/ABL oncoprotein-associated tumors

The study regarding determination of lethal dose

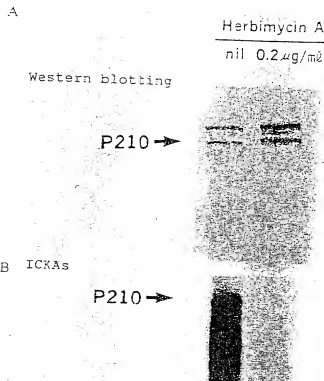


Fig. 2. Suppression of BCR/ABL PTK activity by herbimycin. (A) For the western blotting of BCR/ABL oncoprotein, transformant FDC-P2 cells were cultured for 16 h in the presence or absence of 0.2 µg/ml of herbimycin A, and then cellular protein was isolated. Twenty micrograms of protein were subjected to western blotting. (B) For the immune complex kinase assay (ICKAs), transformant FDC-P2 cells were treated with or without 0.2 µg/ml of herbimycin for 16 h. After this the cell extracts were immunoprecipitated with anti-*c-abl* monoclonal antibody. The resulting immune complexes were analyzed for their autophosphorylation activities.

in DBA/2 mice has revealed that LD<sub>50</sub> of a single i.p. injection of herbimycin A is approximately 20 mg/kg body weight. The intraperitoneal injection of herbimycin daily for 5 days at less than 2.0 mg/kg body weight was well tolerated with no obvious toxicity. A marked reduction in subsequent tumor formation and a prolongation in the survival rates were evident following herbimycin treatment at doses of 1.0–2.0 mg/kg body weight in mice inoculated with greater than  $1 \times 10^5$  of transformant cells (Fig. 4(A)). Furthermore, the i.p. administration with herbimycin A at 2.0 mg/kg body weight completely protected against tumor formation and brought about cures in all mice inoculated with  $1 \times 10^4$  cells, whereas the i.p. administration at 1.0 mg/kg protected against tumor formation in two out of five mice (Fig. 4(B)). In contrast, P815 cells and P388 cells, which were not sensitive to herbimycin A *in vitro*, were transplantable into syngeneic DBA/2 mice, and all mice inoculated i.p. with amounts of greater than  $1 \times 10^4$



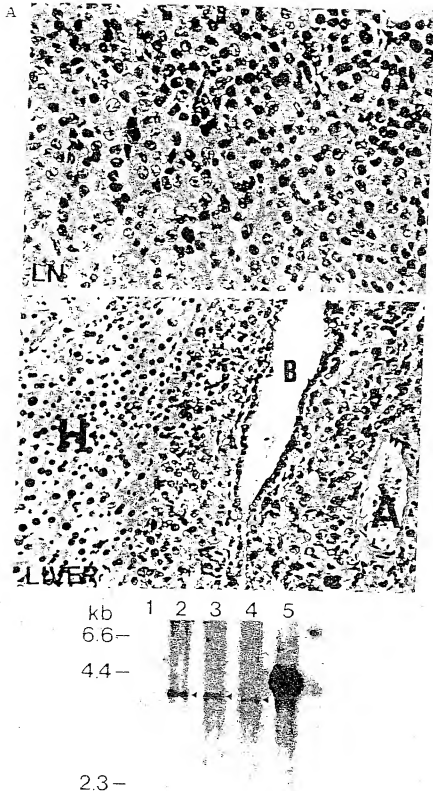


Fig. 3. Histological examination of tumors. (A) Pathological findings of the lymph nodes and the liver. Micrograph of the retroperitoneal lymph node (LN) showing the replacement by tumor cells. Small polygonal cells with prominent nuclei are evident (HE,  $\times 350$ ). Micrograph of the liver: tumor cells are observed throughout the portal tract (HE,  $\times 200$ ). B, bile duct; A, hepatic artery; H, normal hepatocytes. (B) Southern blot analysis revealed the presence of the *bcr/abl* gene. High-molecular weight DNAs isolated from tissues were digested with *Hind*III and were subjected to the Southern blot using 3' *M-bcr* genomic DNA probe. Lane 1, parental FDC-P2 cells; lane 2, transformed FDC-P2 cells; lane 3, lymph nodes; lane 4, liver; lane 5, human placenta DNA. Arrows indicate the signals derived from *bcr/abl* gene.

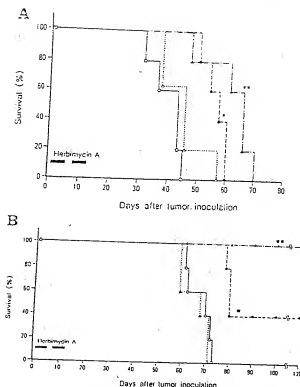


Fig. 4. Therapeutic effects of herbimycin A on DBA/2 mice inoculated with BCR/ABL-transformed FDC-P2 cells. Five DBA/2 mice per group were administered i.p. with herbimycin A (0.1 mg/kg body weight,  $\Delta$ — $\Delta$ ; 1.0 mg/kg body weight,  $\bullet$ — $\bullet$ ; 2.0 mg/kg body weight,  $\blacktriangle$ — $\blacktriangle$ ) on days 1–5 and 8–12 after i.p. inoculation of  $1 \times 10^6$  tumor cells (A) or  $1 \times 10^4$  tumor cells (B). Control groups ( $\circ$ — $\circ$ ) were administered with vehicle only. ■, ■■: significant,  $p < 0.001$  (Student's  $t$ -test).

cells died within 12 weeks. In this case, herbimycin A administration had no effect on their survival rates.

### Discussion

PTK activity has been demonstrated to be associated with stimulatory effects of many growth factors and with transforming oncogenes such as *src*, *abl*, *ros*, *fps*, *yes*, and *fes*. These findings indicate that a variety of PTK may play a critical role in the proliferation and differentiation of cells [22] as well as in some malignant transformations of cells, where  $Ph^+$ -positive leukemia is representative in human malignancies. Until now, several tyrosine kinase inhibitors including genistein, erbstatin and lavendustin have been developed on the basis of inhibition of EGF-associated tyrosine kinase. In contrast to these inhibitors of receptor-type tyrosine kinase,

herbimycin A, which was isolated from the cultured filtrate of *Sirepomyces* sp. MH237-CF-8, was capable of causing a reversion of the transformed Rous sarcoma virus-infected rat kidney cell to normal morphology at modest concentrations via reduction of  $p60^{src}$  PTK activity [15]. Furthermore, we previously demonstrated that herbimycin A preferentially suppressed  $Ph^+$ -positive leukemia cell growth and BCR/ABL oncoprotein-associated transformed murine cells via the inhibition of BCR/ABL PTK activity [12].

In this study, we evaluated the *in vivo* efficacy of herbimycin A in a murine model bearing BCR/ABL oncoprotein-expressing transformed cells. The transformed FDC-P2 cells by a transfection of BCR/ABL grew autonomously *in vitro* and were tumorigenic in syngeneic DBA/2 mice, in which the i.p. inoculation of transformant FDC-P2 cells showed the induction of massive swelling of abdominal lymph nodes and the infiltration of abdominal organs and brought about fatal outcomes in mice. In this murine model bearing the BCR/ABL-associated tumors, the i.p. administration of herbimycin A at concentrations of less than 2 mg/kg body weight for 5 days consecutively for 2 weeks significantly prolonged the survival times of the mice when they were inoculated with more than  $1 \times 10^6$  tumor cells and, furthermore, completely protected against *in vivo* tumor formation in mice that received  $1 \times 10^4$  cells with no overt side effects. Similarly, Honma has previously described the *in vivo* antitumor effect of herbimycin A in the murine model inoculated with highly *v-abl*-expressing murine myeloid cell lines, CI cells [23]. Recently, it has been shown that herbimycin A also is a potent cytotoxin against malignant human tumors with primitive neural features, and the tumorigenicity in nude mice of these sensitive cell lines can be markedly reduced by systemic or topical administration of herbimycin A without any apparent toxicity to the whole animal [24].

More recently, erbstatin and tyrphostins, derivative synthetics of erbstatin, provided an important insight into future therapeutic developments against various types of cancers with amplified ErbB-1 (EGF-R) and ErbB-2 (Neu) through inhibiting PTK activity [25–27]. Furthermore, it has been noted that PTK blockers from the tyrphostin family can discriminate between normal ABL and transforming ABL proteins [28]. In addition to these studies, our development and study of herbimycin A, and further development of chemical modifications might provide an important insight into future therapeutic development according to a new strategy of *bcv-abl* oncoprotein-targeted therapy against  $Ph^+$ -positive leukemia.

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## NOTES

<sup>1</sup>Editor's note: SEER is a set of geographically defined, population-based, central cancer registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Registry data are submitted electronically without personal identifiers to the NCI on a biannual basis, and the NCI makes the data available to the public for scientific research.

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$$\textcircled{1} - 7 \times 7 \times 7 \\ \times 0.5 \approx 200 \text{ m}^3$$

## DT-Diaphorase Expression and Tumor Cell Sensitivity to 17-Allylamino,17-demethoxygeldanamycin, an Inhibitor of Heat Shock Protein 90

Lloyd R. Kelland, Swee Y. Sharp, Paul M. Rogers, Timothy G. Myers, Paul Workman

$\textcircled{4}$  2 human  
CC lines

**Background:** To our knowledge, 17-allylamino,17-demethoxygeldanamycin (17AAG) is the first inhibitor of heat shock protein 90 (Hsp90) to enter a phase I clinical trial in cancer. Inhibition of Hsp90, a chaperone protein (a protein that helps other proteins avoid misfolding pathways that produce inactive or aggregated states), leads to depletion of important oncogenic proteins, including Raf-1 and mutant p53 (also known as TP53). Given its ansamycin benzoquinone structure, we questioned whether the antitumor activity of 17AAG was affected by expression of the NQO1 gene, which encodes the quinone-metabolizing enzyme DT-diaphorase. **Methods:** The antitumor activity of 17AAG and other Hsp90 inhibitors was determined by use of a sulforhodamine B-based cell growth inhibition assay in culture and by the arrest of xenograft tumor growth in nude mice. DT-diaphorase activity was determined by use of a spectrophotometric assay, and protein expression was determined by means of western immunoblotting. **Results:** In two independent *in vitro* human tumor cell panels, we observed a positive relationship between DT-diaphorase expression level and growth inhibition by 17AAG. Stable, high-level expression of the active NQO1 gene transfected into the DT-diaphorase-deficient (by NQO1 mutation) BE human colon carcinoma cell line resulted in a 32-fold increase in 17AAG growth-inhibition activity. Increased sensitivity to 17AAG in the transfected cell line was also confirmed in xenografts. The extent of depletion of Raf-1 and mutant p53 protein con-

firmed that the Hsp90 inhibition mechanism was maintained in cells with high and low levels of DT-diaphorase. 17AAG was shown to be a substrate for purified human DT-diaphorase. **Conclusion:** These results suggest that the antitumor activity and possibly the toxicologic properties of 17AAG in humans may be influenced by the expression of DT-diaphorase. Careful monitoring for NQO1 polymorphism and the level of tumor DT-diaphorase activity is therefore recommended in clinical trials with 17AAG. [*J Natl Cancer Inst* 1999;91:1940-9]

Benzoquinone ansamycins, such as herbimycin and geldanamycin (Fig. 1), exhibit anticancer activity by binding to heat shock protein 90 (Hsp90), a molecular chaperone, and its homologue GRP94 (1,2). In this interaction, geldanamycin competes with adenosine triphosphate at the N-terminal-binding site

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of Hsp90 (3). The interaction results in the lysosome-mediated degradation of several important oncogenic proteins, including Raf-1, c-ErbB2, and mutant (but not wild-type) p53 (also known as TP53) (4-6). Clearly, this molecular profile offers considerable potential for antitumor activity. However, both herbimycin and geldanamycin have limitations as drug candidates because of poor stability and hepatotoxicity (7). This has resulted in efforts to discover improved synthetic analogues (8).

One such compound, the 17-allylamino-17-demethoxy analogue of geldanamycin (17-allylamino-17-demethoxygeldanamycin: 17AAG) (Fig. 1), has also been shown to bind to Hsp90 (9). Although, in rodent and dog toxicology studies, 17AAG retains some of geldanamycin's toxicity in the liver, gallbladder, and kidney [(10) and National Cancer Institute (NCI) drug data file on 17AAG] it has a better therapeutic index. For example, 17AAG exerts antitumor activity against some human melanoma xenografts at nontoxic doses [NCI drug data file on 17AAG and (11)]. Preclinical pharmacokinetic studies show that pharmacologically active concentrations can be achieved in plasma and tissues [NCI drug data file on 17AAG and (12)] and that the major liver microsomal metabolite (shown in Fig. 1) is 17-amino-17-demethoxygeldanamycin (13). In view of its novel mechanism of action and its good therapeutic index, 17AAG has now entered phase I clinical trials as first-in-class Hsp90 inhibitor under the auspices of the U.S. NCI and the U.K. Cancer Research Campaign (CRC). Recently, the structurally distinct macrocyclic antifungal compound radicicol (Fig. 1) has been shown to bind to Hsp90 and inhibit its activity (14-17).

DT-diaphorase, an obligate two-electron-reducing enzyme [reduced nicotinamide-adenine dinucleotide (phosphate):quinone oxidoreductase; EC 1.6.99.2], catalyzes the reduction of various quinones (18). As a result, cells rich in DT-diaphorase are especially sensitive to quinone-containing bioreductive anticancer agents, such as mitomycin C and the indoloquinone EO9, which act as prodrugs for activation to toxic forms by DT-diaphorase (19-21). Some tumor types (notably, colon and non-small-cell lung cancers) have been shown to contain rela-

tively high levels of DT-diaphorase (22-26). Thus, these cancers may be particularly suitable for treatments that use a DT-diaphorase prodrug approach. Although previous studies (27) have shown that geldanamycin is a substrate for DT-diaphorase, a cell line derived from human colorectal cancer and expressing DT-diaphorase did not appear to be particularly sensitive to geldanamycin. However, it is not known whether cells expressing high levels of DT-diaphorase show altered sensitivity to 17AAG.

The primary aim of this study was to investigate whether DT-diaphorase activity has a role in the sensitivity of human tumor cells to 17AAG. Initially, sensitivity to 17AAG was determined by use of the CRC/Institute of Cancer Research (ICR) panel of 15 human colorectal and 11 ovarian carcinoma cell lines, including some resistant to classical agents. Comparative data were obtained in selected lines for the 17-amino metabolite and the additional Hsp90-binding agents geldanamycin and radicicol. The correlation between sensitivity and DT-diaphorase activity seen in a subset of the CRC/ICR panel (selected to span the range of sensitivity to 17AAG) was then examined and confirmed with data from the NCI panel of 60 human tumor cell lines (28). This led to the hypothesis that high DT-diaphorase expression was a major factor in determining cellular sensitivity to 17AAG but not to geldanamycin or radicicol. To provide further conclusive data, sensitivity to 17AAG was determined in a newly established isogenic pair of cell lines that differ only in the expression of the active NQO1 gene. This pair is composed of the human colon BE line [which contains a disabling point mutation in the NQO1 gene encoding DT-diaphorase (29)] and a subline stably transfected with the NQO1 gene and expressing high levels of functional DT-diaphorase. Finally, evidence that the Hsp90 inhibitory mechanism was retained by 17AAG in colon cell lines expressing high and low levels of DT-diaphorase was obtained by immunoblot analysis of Raf-1, mutant p53, Hsp70, and Hsp90 proteins. The results suggest that determination of patients' NQO1 genotype and of tumor DT-diaphorase activity should be included in the clinical evaluation of 17AAG because variations in these characteristics could affect the toxicity and efficacy of the drug.

## MATERIALS AND METHODS

### Cell Lines

We used panels of human colon and ovarian cell lines. We obtained cell lines from commercial cell culture collections or derived them in-house as described previously (30). In some cases, we used sublines derived from a particular parent line with acquired drug resistance to cisplatin (CH1cisR and A2780cisR ovarian lines) or to doxorubicin (CH1doxR and an SKOV-3 subline stably overexpressing the multidrug-resistance protein MRP1) (30-32). All lines were grown as monolayers in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 2 mM glutamine, and 0.5 µg/mL hydrocortisone in 6% CO<sub>2</sub>/94% air. All lines were free of *Mycoplasma* contamination.

### Drugs and Chemicals

Geldanamycin, 17AAG, and 17-amino-17-demethoxygeldanamycin were supplied by E. Sauvville (NCI). The remaining drugs (herbimycin, radicicol, streptonigrin, and dicoumarol) and chemicals were obtained from Sigma Chemical Co. (Poole, U.K.).

### Growth Inhibition Studies

We used the sulforhodamine B assay as described previously (30-32) for growth inhibition studies. Briefly, we seeded tumor cells into 96-well microtiter plates, allowed the cells to attach overnight, and then added the drug to quadruplicate wells.

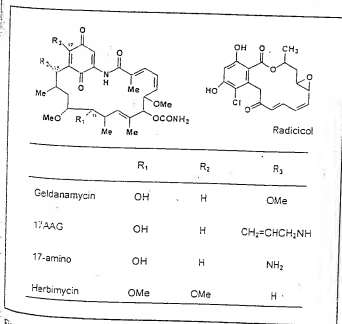


Fig. 1. Chemical structures of geldanamycin, 17-allylamino-17-demethoxygeldanamycin (17AAG), 17-amino-17-demethoxygeldanamycin (17-amino), radicicol, and herbimycin.

plicate wells as indicated. Unless otherwise indicated, we exposed cells to a drug for 4 days. Thereafter, the cell number in treated versus control wells was estimated after treatment with 10% trichloroacetic acid and staining with 0.4% sulfonamide B in 1% acetic acid. The  $IC_{50}$  was calculated as the drug concentration that inhibits cell growth by 50% compared with control growth.

## Stable Transfection of the NQO1 Gene Into the BE Human Colon Carcinoma Cell Line

BE cells contain a point mutation in the NQO1 gene and thus have no functional DT-diaphorase enzyme activity (29). We used the bicistronic expression vector pEFRES-P (33) to express the NQO1 gene in BE cells. Lipofectamine (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) for transfection, and puromycin (0.5 µg/mL) for selection. Resulting clones were screened for DT-diaphorase enzyme activity or protein by an enzyme assay or immunoblotting, respectively (see below). Full details of the vector construction and the biologic properties of the stable transfectants will be published elsewhere (Sharp SY, Kelland LR, Valenti MR, Branton LA, Hobbs S, Workman P: unpublished results). The stable transfectants, designated BE-F397 clone 2 and BE-F397 clone 5, were used in these studies.

## DT-Diaphorase Assay

To determine whether 17AAG was a good substrate for DT-diaphorase, we used the standard cytochrome c assay, as described previously for the bio-reductive indolizone E09 (34) and geldanamycin (27), but replaced menadione with 17AAG as the substrate and intermediate electron acceptor. We assayed extracts of the human colon cell line HT29 or purified human DT-diaphorase protein (from J. Skelly, ICR). For preparation of cell extracts,  $2 \times 10^6$  cells were trypsinized, washed twice in ice-cold phosphate-buffered saline (PBS), and centrifuged (MSE Centaur 1; 1100 rpm for 5 minutes at room temperature). The cell pellet then was resuspended in 0.5–1 mL of lysis buffer (PBS containing 1% Triton X-114 and 500 µM phenylmethylsulfonyl fluoride) and left on ice for 30 minutes. After centrifugation (MSE Microcentrifuge; 12,000 rpm for 5 minutes at room temperature), the supernatant was used for protein determination and the enzyme assay. Results obtained for 17AAG were compared with those for geldanamycin, E09, and streptozin, an excellent substrate for DT-diaphorase (35). For all drugs, the difference in reduction of the menadione substrate in the absence and presence of dicumarol (100 µM), a standard inhibitor of DT-diaphorase, was determined (27).

## Immunoblotting

This analysis was performed as described previously (30–32). Briefly,  $5 \times 10^6$  cells were trypsinized, washed with PBS, and lysed in 100 µL of lysis buffer at 4°C for 1 hour. Lysis buffer contained 10 mL of 150 mM NaCl–50 mM Tris-HCl (pH 7.5), 300 µL of 20 mM phenylmethylsulfonyl fluoride, 2 µL of aprotinin (10 mg/mL stock solution), 2 µL of leupeptin (10 mg/mL stock solution), 100 µL of 10 mM sodium orthovanadate, 100 µL of Nonidet P-40, and 100 µL of 20% sodium dodecyl sulfate (SDS). Lysates were centrifuged (MSE Microcentrifuge; 12,000 rpm for 15 minutes at 4°C), and the resulting protein extracts were separated (30 µg/lane) by SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose filters. Antibodies to Hsp90 and Hsp70 were obtained from StressGen (Victoria, Canada), and antibodies to Raf-1 and p53 (DO1) were from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody to the rat DT-diaphorase (which cross-reacts with human diaphorase) was supplied by R. Knox (previously at CRC/ICR, now at Enzacta Ltd., Salisbury, U.K.). Antibody binding was identified with horseradish peroxidase-labeled secondary antibodies combined with enhanced chemiluminescence reagents (Amersham, Buckinghamshire, U.K.) and autoradiography.

## In Vivo Effects

BE vector control cells and BE-F397 clone 2 cells were established as subcutaneous xenografts by injection of  $5 \times 10^6$  cells into the flanks of adult female athymic nude (nu/nu) mice. The antitumor effect of 17AAG was determined in mice bearing comparably sized tumors (6–8 mm in diameter) derived from these cells. Animals were randomly assigned to receive vehicle alone (five or six mice) or 17AAG (five animals; dose schedule = 80 mg/kg per day in 10% dimethyl sulfoxide and 90% egg phospholipid by intraperitoneal injection on days 1–4 and days 7–11). Before this clinical formulation was available, 17AAG was administered to mice bearing HT29 xenografts in 10% dimethyl sulfoxide–40.5% Tween 20–90% NaCl, with a dose schedule of 80 mg/kg per day on days 0–3 and

days 6–10. This dose and schedule were derived from previously performed experiments [NCI drug data file on 17AAG and (11)].

Tumor size was determined twice weekly by caliper measurements, and tumor volumes were calculated (volume =  $[a \times b^2 \times \pi]/6$ , where  $a$  and  $b$  are orthogonal tumor diameters). Tumor volumes were then expressed as a percentage of the volume at the start of treatment (relative tumor volume). The effect of the drug was determined by the growth delay, i.e., the difference in days required for the volume of tumors in control and treated animals to double. All procedures involving animals were performed within the guidelines set out by the Institute's Animal Ethics Committee and the United Kingdom Coordinating Committee for Cancer Research's *ad hoc* Committee on the Welfare of Animals in Experimentation (Neoplasia [36]).

## Statistical Analyses

Where indicated, errors are presented as standard deviation ( $n \geq 3$ ). Correlation tests and linear regression analyses were computed with SAS JMP (SAS Institute, Cary, NC). We assessed correlations with a Spearman calculation for the CRC/ICR panel and with a Pearson calculation for the NCI panel. Although the Spearman statistic is technically more robust, the Pearson statistic was used for correlations in the NCI panel for historic continuity. The likelihood test for linear model comparison was performed with S-Plus (Mathsoft, Seattle, WA). All  $P$  values are two-sided.

## RESULTS

### In Vitro Growth Inhibition

The *in vitro* growth inhibition properties of geldanamycin, 17AAG, and radicicol against panels of human colon (11 lines) and ovarian (11 lines) carcinoma cell lines are shown in Table 1. A. The  $IC_{50}$  value for 17-amino-17-demethoxygeldanamycin, the major metabolite of 17AAG, is also included for some lines. In most cell lines, all four compounds potently inhibited growth, with  $IC_{50}$  values of less than 2.5 µM. Notably, one ovarian cell line (the 41M line) was relatively resistant ( $IC_{50} > 2.5$  µM) to all four Hsp90-interactive compounds. On average, geldanamycin was the most potent agent (mean  $IC_{50}$  = 30.1 nM), with similar values obtained for 17-amino-17-demethoxygeldanamycin (mean  $IC_{50}$  = 47 nM in a subset of nine cell lines). 17AAG showed intermediate potency (mean  $IC_{50}$  = 220.4 nM), and the least potent agent was radicicol (mean  $IC_{50}$  = 587.4 nM).

Bar graphs showing the  $IC_{50}$  values (Fig. 2) reveal some interesting differences in the patterns of response for geldanamycin, 17AAG, and radicicol. Notably, some cell lines (e.g., BE and LoVo colon cells) are relatively resistant to 17AAG but not to geldanamycin (or radicicol). In contrast, the colon cell lines LS174T and KM12 were relatively resistant to geldanamycin but not to 17AAG. We have compared patterns of response for 25 cell lines (excluding 41M because this line was resistant to all compounds) by use of the Spearman analysis. Positive, but not statistically significant, correlations were observed between geldanamycin and radicicol ( $r = .36$ ;  $P = .08$ ) and between geldanamycin and 17AAG ( $r = .33$ ;  $P = .11$ ). There was, however, no correlation between 17AAG and radicicol ( $r = -.03$ ;  $P = .72$ ). Results indicate relatively distinct patterns of response for the three compounds. 17-Amino-17-demethoxygeldanamycin was studied in only a few lines in the panel. With the exception of LS174T colon cells, which are relatively resistant to geldanamycin and more sensitive to the 17-amino metabolite, the two compounds behaved similarly across the panel.

### Activity in Acquired Anticancer Drug-Resistant Cell Lines

The *in vitro* potencies of geldanamycin, 17AAG, and radicicol have also been evaluated in various anticancer drug-resistant

Table 1. *In vitro* human tumor cell growth inhibitory activity of 17AAG and other heat shock protein inhibitors

**A. Summary of growth inhibition (drug concentrations that inhibit growth by 50% ( $IC_{50}$ )) of geldanamycin, 17AAG, radicicol, and 17-Amino against the CRCR panel of human colon and ovarian tumor cells.\***

Cell line	Geldanamycin	17AAG	Radicicol	17-Amino
<b>Colon</b>				
BE	19.3 ± 3.1	773 ± 30.6	190	18
HT29	46.7 ± 9	8.9 ± 2.9	3100	6.3 ± 1.8
COL205	3.8	7.2	1400	ND
DLD-1	78	140	290	ND
HCA-7	1.8	72	120	ND
HCC2998	98	78	650	ND
HCT15	83	490	280	ND
HCT15h	67	99	240	ND
HT29	11.3	13.5	390	17
KM12	54	9	135	ND
LIM1215	9	77	100	20.5
LS174T	245	78.5	780	33.5
LoVo	21.9	1130	360	42
MAWI	6.2	58	1850	ND
SW620	3.1	68	110	ND
<b>Ovarian</b>				
A2780	11.5	12	115	43
CH1	104.5	1055	325	190
HXA2	47	670	2500	ND
IGR1	94	92	295	ND
OVCAR-3	5	58	69	ND
OVCAR-4	9.6	295	540	ND
OVCAR-5	88	40	660	ND
OVCAR-8	15.5	67	230	ND
PXN94	84	43	1450	ND
SKOV-3	46	76	395	58
4IM	>2500	5200	2350	>2500

**B. Growth inhibitory properties of geldanamycin, 17AAG, and radicicol against anticancer drug-resistant human tumor cell lines.\***

Cell line	Geldanamycin	17AAG	Radicicol
A2780	7.1	2.4	115
A2780cisR	9.1	1.9	270
RF	1.3	0.8	2.3
CH1	49	960	325
CH1cisR	17	1220	88
CH1doxR (Pgp)	0.35	1.3	0.27
RF	2500	>2500	565
RF	51	>2.6	1.7
SKOV-3 (Puro)	97.3	46	380
SKOV-3 S2 (MRP)	337	142	280
RF	3.5	3.1	1.0

\*17AAG = 17-allylamino-17-demethoxygeldanamycin; 17-Amino = 17-methyl-17-demethoxygeldanamycin; CRC = Cancer Research Campaign; ICR = Institute of Cancer Research; MRP = multidrug-resistance protein; Pgp = glycoprotein; puro = puromycin; ND = not done; RF = resistance factor (parental or -transfected line/parent/vector control line).  
†Data are either the mean ± standard deviation (n = 3) or the mean of two determinations.

blines. These lines possess acquired resistance to cisplatin (cisR lines) or to doxorubicin through overexpression of P-glycoprotein (doxR line) or of MRP1 (SKOV-3 S2) (Table 1, B). Although little cross-resistance to geldanamycin was observed in the cisplatin-resistant cell lines, geldanamycin was markedly more potent in the P-glycoprotein-overexpressing cell lines and in the MRP1-overexpressing cell lines than in the parent lines, suggesting that geldanamycin is a substrate for these multidrug-

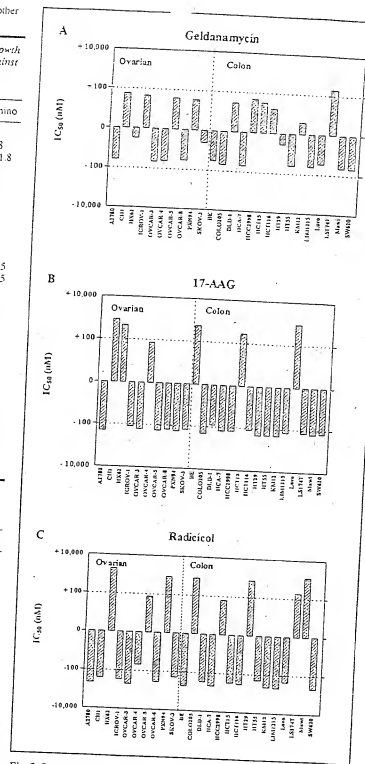


Fig. 2. Patterns of *in vitro* growth inhibition response across 25 human colon or ovarian carcinoma cell lines (as indicated) for geldanamycin (A), 17-allylamino-17-demethoxygeldanamycin (17-AAG) (B), and radicicol (C). Results are displayed as the extent to which the  $IC_{50}$  value (mean drug concentrations that inhibit growth by 50% for a 96-hour exposure) for a given cell line was greater or lower than the mean  $IC_{50}$  calculated for the entire panel. Values for the mean  $IC_{50}$  across the whole panel were as follows: 50.1 nM for geldanamycin, 220.4 nM for 17-AAG, and 387.4 nM for radicicol.

resistant efflux proteins. The picture is rather less clear for 17AAG because the parental CH1 ovarian cell line is relatively resistant to 17AAG, although there is at least a 2.5-fold cross-resistance to 17AAG in CH1doxR. The level of cross-resistance



for geldanamycin and 17AAG is similar in the MRP-overexpressing ovarian line. Like geldanamycin, 17AAG retains full activity in the cisplatin-resistant lines.

#### Growth Inhibition and DT-Diaphorase Enzyme Activity

Because geldanamycin and 17AAG are quinone-based compounds and BE cells have a disabling point mutation in the NQO1 gene (29), the lack of DT-diaphorase activity in these cells could be involved in their surprisingly high relative resistance to 17AAG and low relative resistance to geldanamycin. To explore this possibility, we measured DT-diaphorase enzyme activity and  $IC_{50}$  values for geldanamycin, 17AAG, and radicicol in 11 cell lines (selected from those shown in Table 1), with a broad spectrum of responses to these compounds (Fig. 3). A statistically significant negative Spearman correlation was apparent for 17AAG ( $r = -.81$ ;  $P = .002$ ). Cells with marginal DT-diaphorase levels were relatively resistant to 17AAG, but there was no statistically significant correlation between sensitivity to geldanamycin or radicicol and DT-diaphorase levels ( $P = .33$  and  $.76$ , respectively). Thus, we have identified the potential for a causal link between expression of DT-diaphorase and sensitivity to 17AAG, but not geldanamycin, in the CRC/ICR panel of colorectal and ovarian cell lines.

We then repeated this analysis with the NCI panel of 60 cell lines, which are derived from a diverse group of human cancers (28). We have reported previously the DT-diaphorase activities for this panel of cells (23) as the logarithmically transformed values that are normally used for analysis in the NCI panel. We used correlation tests to explore the hypothesis that DT-diaphorase levels could be directly responsible for the sensitivity differences observed among the cell lines. The Pearson correlation coefficient indicated a weak positive relationship between DT-diaphorase expression and sensitivity to 17AAG ( $r = .11$ ). The correlation between DT-diaphorase and geldanamycin was also weak, with possibly a negative trend ( $r = -.15$ ). Neither correlation was statistically significant ( $P = .43$  and  $.24$ , respectively). We then tested the hypothesis that, although DT-diaphorase activity may not predict sensitivity to 17AAG directly, it might explain why some cell lines are more sensitive to 17AAG than to geldanamycin. We tested this hypothesis by comparing the following two linear regression models: 1) 17AAG sensitivity = geldanamycin sensitivity + error, and 2) 17AAG sensitivity = geldanamycin sensitivity + DT-diaphorase activity + error. Because 17AAG and geldanamycin are reasonably well correlated ( $r = .50$ ;  $P < .001$ ), both models fit the data well. However, more important, inclusion of DT-diaphorase caused a statistically significant ( $P = .03$ ) improvement in the fit as measured with a likelihood ratio test (analysis of variance by use of the F statistic). Thus, DT-diaphorase is a statistically significant factor when the sensitivity patterns of 17AAG are compared with those of geldanamycin. Addition of multidrug resistance protein status, as measured functionally by rhodamine efflux, did not improve the above model (data not shown).

#### Activity in Isogenic BE Colon Cell Lines That Contain or Lack the Active NQO1 Gene

To more directly investigate the role of DT-diaphorase in mediating the cytotoxicity of 17AAG, we stably transfected the BE cell line with the NQO1 gene encoding DT-diaphorase. As shown by immunoblotting, the resulting BE-F397 clone 2 and

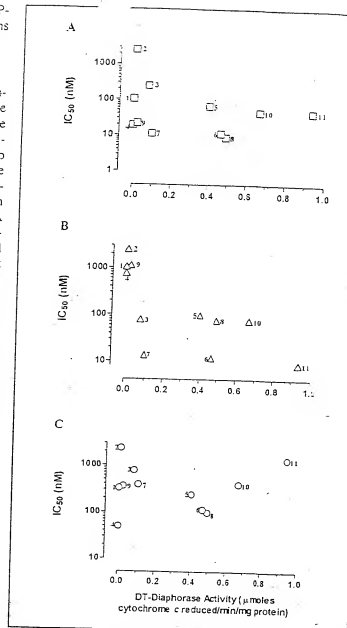


Fig. 3. Relationship between *in vitro* growth inhibition and DT-diaphorase expression for heat shock protein 90 inhibitors. The results are displayed as a plot of  $IC_{50}$  (mean drug concentrations that inhibit growth by 50% in a 96-hour exposure) versus DT-diaphorase enzyme activity and DT-diaphorase enzyme activity for geldanamycin (A), 17-allylamino-17-demethoxygeldanamycin (B), and radicicol (C) in the following 11 human tumor cell lines: 1 = CH1 (ovarian); 2 = 41M (ovarian); 3 = LS174T (colon); 4 = BE (colon); 5 = HCT116 (colon); 6 = A2780 (ovarian); 7 = HT53 (colon); 8 = LIM1215 (colon); 9 = LoVo (colon); 10 = SKOV-3 (ovarian); and 11 = HT29 (colon).

the naturally high DT-diaphorase-containing colon line HT29 possess similar levels of DT-diaphorase protein (unpublished results). Enzyme activity data supported the immunoblotting observations. Values (measured as the decoumarol-inhibitable reduction of menadione and expressed as micromoles of cytochrome *c* reduced per minute per milligram of protein) are as follows: BE vector control cells, unmeasurable activity ( $P < .002$ ); BE-F397 clone 2,  $1.4 \pm 0.5$ ; BE-F397 clone 5,  $1.3 \pm 0.2$ ; and HT29,  $0.94 \pm 0.2$ . This HT29 activity is similar to the activity obtained previously with the same assay (27). Functional validation of the model was provided by the observation

Fig. 4. Shock 1 control symbol (17AAG) compared to radicicol. The DT-diaphorase level of 17AAG is shown.

that introduction of the DT-diaphorase gene into BE cells substantially enhanced the potency of streptonigrin, an excellent DT-diaphorase substrate and bioreductive agent. The degree of potentiation correlated with DT-diaphorase levels and activity (117-fold potentiation in BE-F397 clone 5 and 142-fold potentiation in BE-F397 clone 2). Further details will be published elsewhere.

Dose-response curves for geldanamycin and 17AAG in BE vector control cells and BE-F397 clone 2 are shown in Fig. 4, A. Although the two lines showed similar sensitivity to geldanamycin, BE vector control cells lacking DT-diaphorase were markedly less sensitive to 17AAG. The degrees of potentiation (in terms of  $IC_{50}$  values) for geldanamycin, 17AAG, 17-amino-17-demethoxygeldanamycin, radicicol, and herbimycin observed when DT-diaphorase was introduced into the BE colon cell line are shown in Fig. 4, B. Notably, a 32-fold potentiation

was observed with 17AAG, whereas a less than threefold potentiation was observed for all other compounds evaluated. In a second test of the effect of DT-diaphorase on the growth inhibitory properties of these compounds (Fig. 4, B), HT29 colon cells (naturally high in DT-diaphorase activity) were compared with BE parent cells (no measurable DT-diaphorase activity). Results generally mirrored those results observed with the isogenic-transfected pair of BE lines, with only 17AAG, of the Hsp90 inhibitors tested, showing a marked DT-diaphorase-mediated differential effect (87-fold potentiation). It is of interest in this pair of lines that HT29 cells had a strikingly greater sensitivity to radicicol than did BE cells, an effect not seen with the isogenic BE cell line pair.

#### Reduction of 17AAG by Purified Human DT-Diaphorase

Having demonstrated a potentially important role for DT-diaphorase in cellular sensitivity to 17AAG, we used a menadi-one substrate replacement assay as described previously (27,34) to determine the ability of this agent, geldanamycin, and 17-amino-17-demethoxygeldanamycin to act as substrates for purified human DT-diaphorase (Table 2). Streptonigrin (35), an excellent substrate for DT-diaphorase, was also included in the comparison. We found that 17AAG was a reasonable substrate for DT-diaphorase, but it is not appreciably better than geldanamycin or 17-amino-17-demethoxygeldanamycin. This is perhaps surprising in view of the cellular data. The DT-diaphorase-mediated reduction rate was similar for all three analogues, each at a substrate concentration of  $10 \mu M$ . At  $50 \mu M$ , 17AAG and 17-amino-17-demethoxygeldanamycin gave twofold to threefold higher rates than geldanamycin, and the difference was even greater at  $100 \mu M$ . Geldanamycin at  $100 \mu M$  resulted in substrate inhibition, which was not observed with the other two analogues at  $100 \mu M$ . The latter two concentrations, however, are much higher than the pharmacologically relevant range. It also should be noted that all three of the ansamycin analogues gave reaction rates that were substantially lower than rates observed for streptonigrin (Table 2). With the structurally distinct Hsp90 inhibitor radicicol, which lacks a quinone moiety, no reduction was observed.

#### Effects of 17AAG on Hsp90, Hsp70, and Oncogenic Proteins

To determine whether the mode of action of 17AAG was the same in cells expressing low and high levels of DT-diaphorase and to guide the choice of molecular pharmacodynamic markers in the imminent clinical trial, we measured the levels of Raf-1, mutant p53, Hsp90, and Hsp70 proteins in vector control cells and transfected BE cells treated with 17AAG (or geldanamycin). Levels of these proteins 6 and 24 hours after the addition of equitoxic (continuous exposure to  $5\times$  and  $10\times$   $IC_{50}$ ) or equimolar ( $0.15$  and  $0.3 \mu M$ ) geldanamycin or 17AAG are shown in Fig. 5. No change in Hsp90 protein levels was observed. A similar marked reduction, especially at 24 hours, was observed for Raf-1 and p53 proteins in the BE vector control cells and BE-F397 clone 2 cells at equitoxic concentrations. By contrast, an increase in Hsp70 levels was observed. For geldanamycin or 17AAG at equimolar concentrations ( $0.15$  or  $0.3 \mu M$ ), no change in any of the four proteins was observed in the BE vector control cells expressing low levels of DT-diaphorase, consistent with their cellular resistance at these concentrations.

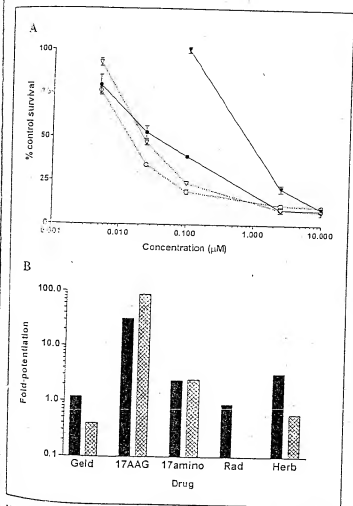


Fig. 4. Effects of DT-diaphorase gene (NQO1) transfection on response to heat shock protein 90 (Hsp90) inhibitors. A) Dose-response curves for BE vector control (open symbols) and BE-F397 clone 2 (filled symbols) for geldanamycin (Geld; open symbols, solid lines) and 17-allylamino-17-demethoxygeldanamycin (17AAG; solid symbols, continuous lines). B) Extent of potentiation in high compared with low DT-diaphorase-expressing cells for the Hsp90 inhibitors geldanamycin, 17AAG, 17-amino-17-demethoxygeldanamycin (17Amino), radicicol (Rad), and herbimycin (Herb). Fold-potentiation = drug concentrations that inhibit growth by 50% ( $IC_{50}$ ) in cells expressing low levels of DT-diaphorase (BE parental or BE vector control line)/ $IC_{50}$  in cells expressing a high level of DT-diaphorase (BE-F397 or HT29 colon line). Solid bars = BE vector control/BE-F397 cells; cross-hatched bars = BE/HT29 cells.

Table 2. Reduction of geldanamycin, 17-allylaminogeldanamycin (17AAG), and 17-amino,17-demethoxygeldanamycin (17-AMN) by purified human DT-diaphorase (at 20  $\mu$ g/mL)\*

Substrate	Reduction of substrate, $\mu$ mol of cytochrome c reduced per minute per mg of protein
Menafladone, 10 $\mu$ M	118.5 $\pm$ 163.7
Streptonigrin, 30 $\mu$ M	206.1 $\pm$ 6.0
Streptonigrin, 25 $\mu$ M	159.1 $\pm$ 9.0
Streptonigrin, 10 $\mu$ M	176.6 $\pm$ 69.5
Geldanamycin, 100 $\mu$ M	1.5 $\pm$ 0.8
Geldanamycin, 50 $\mu$ M	7.2 $\pm$ 3.3
Geldanamycin, 25 $\mu$ M	7.0 $\pm$ 1.0
Geldanamycin, 10 $\mu$ M	4.3 $\pm$ 0.6
17AAG, 100 $\mu$ M	20.5 $\pm$ 2.1
17AAG, 50 $\mu$ M	15.0 $\pm$ 7.9
17AAG, 25 $\mu$ M	8.3 $\pm$ 1.3
17AAG, 10 $\mu$ M	3.6 $\pm$ 0.6
17-AMN, 100 $\mu$ M	17.6 $\pm$ 3.3
17-AMN, 50 $\mu$ M	22.8 $\pm$ 3.0
17-AMN, 25 $\mu$ M	11.7 $\pm$ 4.3
17-AMN, 10 $\mu$ M	6.8 $\pm$ 3.5
Radicalol	ND

\*Values are individual or mean  $\pm$  standard deviation ( $n = 3$ ).

†ND = not detectable at all concentrations tested.

### In Vivo Effects of 17AAG

We determined the effect of 17AAG on the response of the BE vector control cells and BE-F397 cells when grown subcutaneously as solid tumor xenografts in nude mice. 17AAG was administered at the maximum tolerated dose of 80 mg/kg per day intraperitoneally on days 0–4 and days 7–11, a schedule that is active on sensitive xenografts [NCI drug data file on 17AAG and (11)]. The xenograft tumor grown from the transfected BE-F397 cells (Fig. 6, B) was more sensitive than the BE vector control cells (Fig. 6, A). The growth delays, calculated from the time required to reach twice the treatment volume, were 11.4 days for the BE-F397 xenograft and 5.8 days for the vector control. For the HT29 xenograft (and a similar schedule of 80

mg/kg per day intraperitoneally on days 0–3 and days 6–10), a growth delay of 16.6 days was observed (Fig. 6, C). Experiments (not shown) confirmed that the differences in DT-diaphorase expression seen *in vitro* were maintained in the xenograft (data not shown). Thus, the HT29 line with a naturally high level of DT-diaphorase and also the transfected BE-F397 line were more sensitive *in vivo* than the BE vector control cells that have a low level of DT-diaphorase activity.

### DISCUSSION

17AAG is currently entering phase I clinical trial as the first-in-class Hsp90 inhibitor, under the auspices of the NCI and CRC. Treatment with this drug results in the depletion of a number of important oncogenic proteins, including Raf-1, ErbB2, and mutant p53 proteins, from tumor cells (1,4–6,9). In this article, we show that the levels of DT-diaphorase activity in a tumor cell are an important and statistically significant determinant of how well 17AAG will inhibit the growth of that tumor cell. Evidence for this role of DT-diaphorase comes from the following three observations: 1) There was a statistically significant correlation between DT-diaphorase activity and sensitivity to 17AAG for 11 human colon and ovarian cancer cell lines from the CRC/ICR panel. 2) Subsequent interrogation of data from the NCI panel of 60 human tumor cell lines supported the hypothesis that the level of DT-diaphorase activity was a contributory factor in the differences in the sensitivity of tumor cell lines to 17AAG compared with geldanamycin. [In an analogous way, the differences in sensitivity between methotrexate and trimetrexate in the NCI 60 human tumor cell line panel have been explained by differences in the levels of reduced folate carrier protein (37).] 3) Transfection of DT-diaphorase into the BE human colon cancer cell line, thereby creating pairs of isogenic cell lines differing only in DT-diaphorase expression, resulted in a marked increase in 17AAG-induced growth inhibition *in vitro* and an increased response to 17AAG *in vivo*. The degree

Fig. 5. Representative immunoblots for heat shock protein 90 (Hsp90), Raf-1, p53, and Hsp70 (as indicated) in BE vector control or BE clone 2 cells exposed to equitoxic concentrations (5x or 10x drug concentrations that inhibit growth by 30% [IC<sub>30</sub>]) of geldanamycin (0.2 and 0.4  $\mu$ M for 5x and 10x IC<sub>30</sub> in BE vector control cells and 0.1 and 0.2  $\mu$ M for 5x and 10x IC<sub>30</sub> in BE-F397 clone 2 cells, respectively) or 17-allylaminogeldanamycin (17AAG; 7 and 14  $\mu$ M for 5x and 10x IC<sub>30</sub> in BE vector control cells and 0.15 and 0.3  $\mu$ M for 5x and 10x IC<sub>30</sub> in BE-F397 clone 2 cells, respectively). Two fixed concentrations of 17AAG (0.15 and 0.3  $\mu$ M) are also shown for Raf-1 in the BE vector control cells. Cells were exposed to drug for 2 hours and harvested 6 and 24 hours after exposure. Lane 1 = 6-hour incubation of untreated cells; lane 2 = 6-hour incubation in geldanamycin (5x IC<sub>30</sub>); lane 3 = 24-hour incubation in geldanamycin (5x IC<sub>30</sub>); lane 4 = 6-hour incubation in geldanamycin (10x IC<sub>30</sub>); lane 5 = 24-hour incubation in geldanamycin (10x IC<sub>30</sub>); lane 6 = 6-hour incubation in 17AAG (5x IC<sub>30</sub>); lane 7 = 24-hour incubation in 17AAG (5x IC<sub>30</sub>); lane 8 = 6-hour incubation in 17AAG (10x IC<sub>30</sub>); lane 9 = 24-hour incubation in 17AAG (10x IC<sub>30</sub>); and lane 10 = 24-hour incubation of untreated cells. Blots for the BE vector control cells and 17AAG are also shown. Lane 11 = 6-hour incubation of untreated cells; lane 12 = 6-hour incubation in 0.15  $\mu$ M 17AAG; lane 13 = 24-hour incubation in 0.15  $\mu$ M 17AAG; lane 14 = 6-hour incubation in 0.3  $\mu$ M 17AAG; lane 15 = 24-hour incubation in 0.3  $\mu$ M 17AAG; and lane 16 = 24-hour incubation of untreated cells.

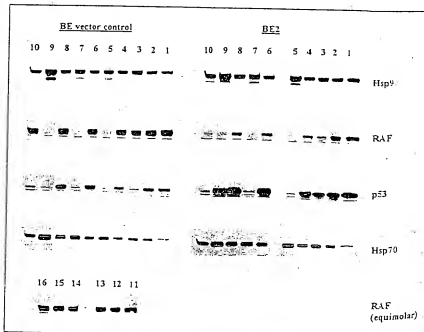


Fig. 6. Xenografts of BE-F397 or HT29 (80 mg/kg; tamors; control; from Fig.

of *in vi* express the high transfect the DT the BE-F397; (27). M demeth 17AAG; (13) and 17AAG; potency diaphorase.

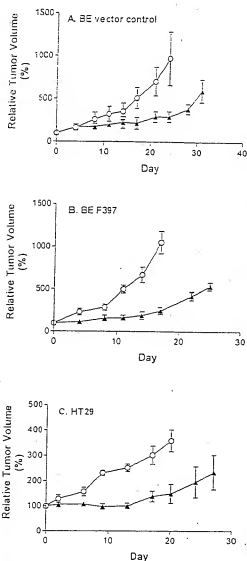


Fig. 6. Effect of DT-diaphorase expression on the response of human tumor xenografts *in vivo* to 17-allylamino-17-demethoxygeldanamycin (17AAG). Tumor growth curves for mice bearing BE vector control (A), BE-F397 clone 2 (B), or HT29 (C) xenografts after treatment with 17AAG. The dosing schedule was 50 mg/kg per day intraperitoneally daily on days 1-4 and days 7-11 for BE tumors and on days 0-3 and days 6-10 for HT29 tumors. ▲ = 17AAG; O = control. Data for relative tumor volumes are the means ( $\pm$  standard deviation) from five animals.

of *in vitro* growth inhibition correlated with the level of enzyme expression, being 32-fold higher in the transfected cell line with the higher levels of DT-diaphorase and 22-fold higher in the transfected cell line with somewhat lower levels. Of interest, the DT-diaphorase effect was not observed with geldanamycin, results in agreement with data from a nonisogenic pair (27). Moreover, the effect was not seen with 17-amino-17-demethoxygeldanamycin, which was identified as the major 17AAG metabolite in human and mouse hepatic preparations (13) and confirmed as such *in vivo* (NCI drug data file on 17AAG). DT-diaphorase activity also appeared unrelated to the potency of radicicol, the structurally distinct Hsp90-binding antibiotic. Indeed, BE cells that express a low level of DT-diaphorase were almost 10-fold more sensitive to radicicol than

were HT29 cells, which express a naturally high level of DT-diaphorase. There was no difference with radicicol in the isogenic transfected BE cell line pair.

The correlation seen between expression of DT-diaphorase activity and sensitivity to 17AAG but not to geldanamycin or radicicol shows that the effect is not generic across all Hsp90 inhibitors or, indeed, across all benzoquinone ansamycins. The precise mechanism by which high levels of DT-diaphorase in tumor cells result in sensitivity to 17AAG is not clear. The observation that DT-diaphorase activity affects tumor cell sensitivity to 17AAG but not to geldanamycin or 17-amino-17-demethoxygeldanamycin is not explicable in terms of their respective behavior as substrates for the purified human enzyme. Although we have demonstrated that 17AAG is a reasonable substrate for human DT-diaphorase, it was not appreciably better than geldanamycin or 17-amino-17-demethoxygeldanamycin, particularly at more relevant drug concentrations. Only at the markedly suprapharmacologic concentrations of 50 and 100  $\mu$ M was 17AAG reduced at a statistically significantly faster rate than geldanamycin. For 17-amino-17-demethoxygeldanamycin, there was no appreciable difference in rate compared with geldanamycin.

Given the close structural similarity of 17AAG, 17-amino-17-demethoxygeldanamycin, and geldanamycin (Fig. 1), it is clear that it is the allyl substitution on the amino group at position 17 that is responsible for the DT-diaphorase effect. Preliminary results with a range of 17AAG analogues are consistent with this observation. We hypothesize that the behavior of the reduction product of 17AAG must differ from the reduction products derived from geldanamycin analogues with other substituents.

The xenograft experiment confirmed that DT-diaphorase-transfected BE-F397 cells were more sensitive than BE vector control cells in a solid tumor *in vivo*. The naturally high DT-diaphorase-containing HT29 xenograft was also more sensitive than the BE vector control xenograft. Dose-response data were not generated in these experiments. However, it seems likely that the differences seen in the *in vivo* xenografts were not as large as those observed in the same lines *in vitro*. One factor that would tend to decrease the contribution of DT-diaphorase levels in the xenograft experiments is the metabolism of 17AAG to the 17-amino derivative, which is the major metabolite in the mouse (13). This could be important because we show in this article that sensitivity to the 17-amino metabolite is not affected by DT-diaphorase. Formation of the 17-amino metabolite is catalyzed by cytochrome P-450, specifically CYP3A4 in human microsomes (13). Thus, we propose that the sensitivity of a given patient's tumor to 17AAG may be affected by the balance between DT-diaphorase and CYP3A4 metabolism. Consequently, we urge that both enzymes (or surrogates thereof) be monitored in the clinical studies that are now under way with 17 AAG.

We determined that 17AAG was operating through the Hsp90 protein to stimulate degradation of the oncogenic client proteins Raf-1 and mutant p53 by use of 17AAG at equitoxic and equimolar concentrations and cells expressing high and low levels of DT-diaphorase. The depletion of client proteins reported previously for both 17AAG and geldanamycin (4-6,9) was seen in cells expressing high and low levels of DT-diaphorase. At equitoxic concentrations of 17AAG or geldanamycin (5x and 10x IC<sub>50</sub>) in the isogenic BE cell lines after 6 hours and, especially, after 24 hours of drug exposure, there was a similar and marked reduction in Raf-1 and mutant p53 proteins. At the fixed

concentrations of 0.15 or 0.3  $\mu$ M 17AAG, which inhibited growth of wild-type NQO1-transfected cells but not BE vector control cells, there was no reduction in Raf-1 or p53 protein in cells with low levels of DT-diaphorase, whereas depletion was seen in the cells with high levels of DT-diaphorase that did respond to these concentrations. Thus, target activity was maintained in the presence of the respective active concentrations of 17AAG, independent of the expression of DT-diaphorase. This rules out the possibility that different target mechanisms operate in cells expressing low and high levels of DT-diaphorase. Rather, DT-diaphorase expression increases the potency of 17AAG via client protein depletion.

In contrast to effects reported in melanoma xenografts after administration of 17AAG (11), no difference in the levels of Hsp90 was observed in our experiments. Hsp90 levels, however, were increased, consistent with the removal of Hsp90-induced transcriptional repression of Hsp70 when Hsp90 is inhibited (38). Again, this effect was seen at equitoxic concentrations of 17AAG in both high and low DT-diaphorase lines, consistent with retention of the Hsp90-binding mechanism.

The high constitutive expression of p53 in BE cells suggests a mutant p53 genotype. Effects on mutant p53 were consistent with cell cycle effects of geldanamycin reported in cell lines expressing wild-type or mutant p53 (39). In our own studies on the A2780 human ovarian carcinoma cell line (wild-type for p53) and a subline stably transfected with the viral p53-inactivating gene HPV6E (40), we found no difference in sensitivity to geldanamycin or 17AAG. Overall, the results indicate that p53 status is unlikely to influence sensitivity to 17AAG.

In summary, although uncertainties remain regarding the precise mechanism involved, our results clearly show that expression of DT-diaphorase can influence a tumor's sensitivity to 17AAG. It is also possible that NQO1 expression could affect toxicity of 17AAG toward normal tissues. There are obvious implications for the clinical evaluation of 17AAG as an anticancer agent because 5%–20% of the population (depending on ethnicity) is homozygous for the genetic polymorphism used in this study, the DT-diaphorase-disabling point mutation in the NQO1 gene present in the BE colon cell line (41). In addition, the expression of DT-diaphorase in human tumors is very variable (25,26), as it is in the cell lines studied herein and elsewhere (22–24). We suggest that, in addition to measuring degradation of oncogenic client proteins and/or an increase in Hsp70 after treatment with 17AAG as potential markers of activity and therapeutic response, NQO1/DT-diaphorase genotype, CYP3A4 status, and also tumor DT-diaphorase levels should be determined. In particular, we propose that these measurements may provide useful indicators of efficacy and/or toxicity and should be considered for the phase I clinical trials of 17AAG that have recently begun under the auspices of the NCI and CRC.

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## NOTES

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tion by reducing the surface expression of KDR on HUVECs, or the affinity or total amount of VEGF binding to KDR on HUVECs. Instead, it appears that the curable effect of SU5416 may be due to residual pool of inhibitor, which is concentrated in cells, that remains associated with cells. The sub-cellular location and kinetics of elimination of the inhibitor are currently under investigation.

# #2843 INHIBITION OF NF- $\kappa$ B BY A NOVEL PROTEASOME INHIBITOR AND ANTI-TUMOR ACTIVITY IN SQUAMOUS CELL CARCINOMA. J B Sunwoo, Z Chen, G Dong, C V Crows-Brown, N Yeh, J Adams, J Mitchell, E Sasville, and C Van Wase. Leukotek, Inc., Cambridge, MA, and National Inst of Health, Bethesda, MD

Squamous cell carcinoma (SCC) of the head and neck has an elevated constitutive activation of the NF- $\kappa$ B transcriptional regulator. We have evidence suggesting that this activation is important for cell survival, tumor development, and protection from ionizing radiation. Activation of NF- $\kappa$ B depends on the proteolysis of the inhibitory protein I $\kappa$ B by the 26S proteasome. In this study, a novel proteasome inhibitor, PS-341 (Leukotek, Inc.), was used to inhibit NF- $\kappa$ B, and its anti-tumor effects were examined in a variety of murine and human SCC cell lines. A 50% inhibition of NF- $\kappa$ B was demonstrated by reporter gene and electrophoretic mobility shift assays at  $10^{-6}$  M concentration. This correlated with anti-proliferation assays, demonstrating an IC<sub>50</sub> of  $10^{-6}$  M. Flow cytometry was used to show that cytotoxicity was also examined *in vivo*, and a significant dose-dependent response was observed. Because exposure to PS-341 induced a cell cycle block at G2/M and was also found to inhibit induction of NF- $\kappa$ B by ionizing radiation, we examined the utility of this compound as a sensitizer to ionizing radiation. We found a 30% increase in radiosensitivity by colony assay after accounting for direct cytotoxic effects of the compound. These results suggest that the use of proteasome inhibitors to target the constitutive activation of NF- $\kappa$ B may be a useful therapeutic strategy in patients with squamous cell carcinoma of the head and neck.

# #2844 RESPONSE OF HUMAN MELANOMAS TO 17-AAG IS ASSOCIATED WITH MODULATION OF THE MOLECULAR CHAPERONE FUNCTION OF HSP90. Angelika Maria Burch, Edward A Sasville, Richard F Camaleri, David J Newman, and Heinz H Flebig. National Cancer Inst, Bethesda, MD, Tumor Biology Ctr, Freiburg, Germany, and Univ of Freiburg, Freiburg, Germany

17-allylamino-17-demethoxygeldanamycin (17-AAG, NSC 330507) is a new antitumor agent identified by the NCI which has entered phase I clinical trials in the US. Antitumor activity of geldanamycins has been described to result from degradation of signaling proteins and nuclear hormone receptors by binding their molecular chaperone Hsp90. In this study, two human melanomas xenografts, the 17-AAG sensitive MEXF 276 (IC<sub>50</sub> = 6%), the resistant MEXF 514 (IC<sub>50</sub> = 60%), and cell lines derived thereof, were chosen to elucidate 17-AAG effects on its potential target Hsp90 and down-stream effector proteins in a time and concentration dependent manner. Tumor tissues were collected after 48h, 72h, and 10d under 17-AAG treatment (at MTD = 80mg/kg, for 2x, Qd5x). Cell lines were exposed to drug concentrations which cause total growth inhibition in MEXF 276 (176L, 10  $\mu$ M in MEXF 514L cells). By using immunohistochemistry and Western blot analysis we found Hsp90 abundantly expressed in 17-AAG responsive MEXF 276 tumors, but at lower levels in resistant MEXF 514 and in normal tissues. Moreover, whilst 17-AAG treatment did not affect Hsp90 expression in MEXF 514, it caused a rapid decline of Hsp90 in MEXF 276 cells. In latter, this was accompanied by translocation of Hsp90 from cytoplasm and nuclei to cell membrane. In contrast, Hsp72 levels were not changed in either melanoma. As a result of Hsp90 depletion in MEXF 276L cells, down-regulation of Raf-1 and HER-2/neu was observed 8h after drug addition. In MEXF 276 tissues, decrease of Hsp90 was further associated with occurrence of apoptosis. The apoptotic index rose from 9% (48h) over 12% (72h) to 45% (10d) under drug treatment. Our data suggest that the efficacy of 17-AAG is related to its ability to inhibit Hsp90 chaperone function.

# #2845 ANTICANCER EFFECTS OF LIPOSOME-ASSOCIATED L AND D TETRAISOMERS OF ET-18-OC<sub>3</sub>. I Ahmad, R P Masters, J Nguyen, J D Juchysky, A S Janoff, and E Mayhew. The Liposome Company (TLQ), Princeton, NJ

TLQ-ELL-12 is a liposome based formulation of ET-18-OC<sub>3</sub> (1- $\alpha$ -octadecyl-2- $\alpha$ -methyl-sn-glycero-3-phosphocholine), and is currently in Phase I clinical trials. The L isomer of ET-18-OC<sub>3</sub> is the active ingredient of ELL-12. We have previously shown the therapeutic efficacy of ELL-12 against several experimental mouse tumors. The aim of the present investigation was to determine any difference in toxicity or therapeutic efficacy of ELL-12 when formulated with L or D stereoisomers of ET-18-OC<sub>3</sub>. The L isomer liposome formulation (L-ELL-12) significantly reduced toxicity compared to the D isomer liposome formulation when administered once daily, i.v. x 5. L and D isomer formulations of ELL-12 were found to be equally effective in prolonging mean survival time against P388 murine leukemia. However, the L isomer liposome formulation, when administered against established B16/F10 lung tumors, significantly ( $p < 0.05$ ) reduced the mean number of tumor nodules compared to control or the D isomer liposome formulation. These studies indicate that ELL-12 formulated with the L isomer of ET-18-OC<sub>3</sub> is less toxic and more effective against B16/F10 tumor than the D isomer liposomes.

# #2846 THE APOPTOTIC EFFECT OF LONG-CHAIN FATTY AMINES ON HUMAN PANCREATIC CANCER CELLS IS MEDIATED BY SIGNALING PATHWAYS INCLUDING MAPK FAMILY AND CASPASES. Mizukami Yusuke, H. Ura, T. Obara, T. Iizawa, N. Yanagawa, S. Tanno, Y. Fujimoto, and Y. Kohgo. Asahi Kasei Med. Co., Hachioji, Japan

Farnesyl transferase inhibitor (FTI) is usually ineffective in K-ras transformed cells. However, we have shown that farnesylamine (FA), one of FTI could induce apoptosis in K-ras transformed fibroblasts and human pancreatic cancer cell lines (MD Carcinogenesis, 1998). Therefore, we speculated that FA may have another apoptotic mechanism in addition to the inhibition of farnesylation. Considering the chemical formula of FA, the "long-chain fatty amine (LFA)" structure may play a critical role for this mechanism. In this experiment, we used oleoylamine (OA) as LFA and examined the signaling pathways to induce apoptosis in K-ras transformed fibroblasts and human pancreatic cancer cell lines. In both cells, apoptosis was induced by OA and JNK activity was increased as well as by FA, but not in parent fibroblasts (NIH3T3). Although the OA-induced apoptosis was caspase-3 dependent, caspase inhibitors did not affect JNK activation. The blockage of JNK activity by dominant negative mutant significantly abrogated the cytotoxic effect of OA and DNA laddering. OA did not act as FTI, but decreased the upregulated ERK activity. In contrast to indispensable effect of JNK in OA-induced apoptosis, attenuated ERK activity alone was not sufficient, but might be required, because MEK inhibitor PD98059 alone did not induce apoptosis. The kinase activity of Akt, which transduce p21 ras mediated survival signaling, resulted in no marked change. Multiple signaling pathways including JNK, ERK, and their downstream caspases mediate the apoptosis and might be altered, at least in part, in FA-induced selective cytotoxicity on K-ras mutant cells.

# #2847 PHARMACOLOGICAL INDUCTION OF PHOSPHATIDYLINOSITOL ACCUMULATION IS ASSOCIATED WITH CYTOLYSIS OF NEOPLASTIC CELLS. Robert E Finney, E Nudelman, S A Shaffer, T White, S Bursten, L L Leer, H Wang, D Waggoner, J W Singer, and R A Lewis. Cell Therapeutics, Inc, Seattle, WA

De novo phospholipid biosynthesis is required for growth of tumor cells. Here, we demonstrate that phospholipid biosynthesis through phosphatidic acid (PA) in neoplastic cells can be exploited for development of cytotoxic anti-cancer agents. PA is a key intermediate for biosynthesis of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) through a diacylglycerol (DAG) intermediate and for biosynthesis of the anionic phospholipids, cardiolipin (CL) and phosphatidylinositol (PI), through a cytidinediphosphate-DAG intermediate. In addition to de novo PA production from lysophosphatidic acid (LPA), production of PA by phospholipase D has been given attention as the effects of certain cytotoxic agents (e.g. ras, pif, and src) and growth factors (e.g. PDGF, EGF, FGF, Insulin). CT-2584, a cancer chemopreventive drug candidate currently in Phase II clinical trials, decreased utilization of PA for PC biosynthesis and increased PA utilization for PI biosynthesis. A two- to three-fold increase in PI was observed in tumor cell lines derived from breast, lung and prostate, was associated with cytotoxic concentrations of CT-2584, and occurred well prior to cytotoxicity in the tumor cell lines. In contrast, cytotoxic concentrations of cisplatin did not induce accumulation of PI, indicating that PI elevation by CT-2584 was not a general consequence of chemotherapy-induced cell death. Consistent with this mechanism of action, proparganol, an inhibitor of phosphatidic acid phosphatase and PC biosynthesis, was also cytotoxic to tumor cell lines, induced PI accumulation, and was synergistic with CT-2584 in cytotoxicity assays. As expected from the biophysical properties of anionic phospholipids on cellular membranes, CT-2584 cytotoxicity was associated with disruption and swelling of endoplasmic reticulum and mitochondria. We conclude that CT-2584 effects a novel mechanism of action involving modulation of phospholipid metabolism in cancer cells.

# #2848 THE EFFECTS OF LYSPHOSPHATIDYLCHOLINE ON TNF- $\alpha$ PRODUCTION INDUCED BY LIPOSOMAL ET-18-OC<sub>3</sub>. Marina Y Pushkareva, Andrew S Janoff, and Eric Mayhew. The Liposome Co., Inc, Princeton, NJ

The incorporation of 1- $\alpha$ -octadecyl-2- $\alpha$ -methyl-sn-glycero-3-phosphocholine (ET-18-OC<sub>3</sub>) into optimized liposomes (ELL-12) overcomes the non-specific hemolytic effects of ET-18-OC<sub>3</sub> while maintaining or enhancing anti-cancer efficacy. ELL-12 is currently in Phase I clinical trial. We showed previously that *in vitro* ELL-12 induced growth inhibition is associated with a time- and dose-dependent production of tumor necrosis alpha (TNF- $\alpha$ ). As lysophosphatidylcholine (lysoPC) has been shown to regulate the growth inhibiting effects of ELL-12, it was of interest to determine the role of lysoPC on ELL-12-induced growth inhibition by U-937 cells. We treated U-937 cells with different concentrations of ELL-12 and lysoPC for various times. Maximum of TNF- $\alpha$  production (0.78  $\pm$  0.17 ng per 10<sup>6</sup> cells) was observed after 48 hours of incubation of U-937 cells with 3.4  $\mu$ M ELL-12. LysoPC prevented induction of TNF- $\alpha$  production in dose-dependent manner. For example, 20  $\mu$ M of lysoPC completely prevented TNF- $\alpha$  production at 48 hours, whereas 2  $\mu$ M lysoPC produced 50% inhibition. The effects on TNF- $\alpha$  production were not directly coupled to the effects of lysoPC on reduction of ELL-12-induced growth inhibition, since 2  $\mu$ M lysoPC did not significantly affect ELL-12-induced growth inhibition. ET-18-OC<sub>3</sub> and lysoPC share structural similarity and have common cellular targets including inhibition of de novo phosphatidylcholine synthesis. The possible mechanism of inhibition of ELL-12-induced TNF- $\alpha$  production by lysoPC will be discussed.

(4) - Clinical  
melanoma

Notes on 17-AAG clinical trials from ASCO meeting, May 2001, San Francisco  
L. Fritz

1. Banerji et al (Workman group, UK). (Abstract 326)

Enrolled 21 patients; 6 melanoma (most of any type). 2 patients showed stable disease (both melanoma, at 320 mg/). Drug comes as 25 mg/ml in DMSO.

PD measurements: HSP70 increased  
Raf decreased  
In one melanoma patient, CDK4 went down and HSP70 went up (no Raf in that tumor)

PK: at 320 mg/m<sup>2</sup>: Cmax = 5-10 uM

Has good xenograft data.

No myelotoxicity even at peak doses.

2. Wilson et al (NIH arm of the study) (Abstract 325)

Richard Wilson presented the poster, but has returned to Northern Ireland. Jean Grem now heads the NIH arm of the clinical trial.

Patients entered:

Colorectal	6
Pancreatic	5
Renal	2
Various	1 each

Total 18 patients

Wilson says Len Neckers was wrong - no lung cancer response was seen - in fact, n900 lung cancer patient has been entered at NIH.

They have seen two patients with stable disease: 1 colorectal and 1 renal. But, Wilson says you see these types of patients stabilize spontaneously.

PD measurements:

Had good degradation of Lck and Raf-1 in PBL's (by day 2) also, HSP70 up - all by Western (done by Neckers).

Regarding who at CTEP is in charge of the 17-AAG project:

3



Louise Grachow      Runs Investigative Drug Branch in CTEP

Susan Arbuck      Runs Developmental Chemotherapy Section (runs it, or is in it?)  
Reports to Grachow. Is directly responsible for 17-AAG. Arbuck is the  
person we should contact

Dale Shoemaker      He was mentioned, but I'm not sure what his role is.

Sherry Ansher      Interacts with companies for CTEP. Jean Grém described  
her as "more like a lawyer".

3. Munster et al (MSKCC trial data) (Abstract 327)

Pam said she'd send us the slides that comprised her poster

4. Also spoke with the clinician who runs the Mayo Clinic arm of the 17-AAG trial. He said they are having trouble reliably measuring Lck and Raf in their blood samples (Neckers had better gels – but said not all samples had Raf either). NCI seemed to have the best PD measurements of all the sites.

## Advances in Brief

17-Allylamino-17-demethoxygeldanamycin Induces the Degradation of Androgen Receptor and HER-2/*neu* and Inhibits the Growth of Prostate Cancer Xenografts<sup>1</sup>

David B. Solit, Fuzhong F. Zheng, Maria Drobnjak, Pamela N. Münster, Brian Higgins,<sup>2</sup> David Verbel, Glenn Heller, William Tong, Carlos Cordon-Cardo, David B. Agus,<sup>3</sup> Howard I. Scher, and Neal Rosen\*

Program in Cell Biology [D. B. S., F. F. Z., P. N. M., N. R.], and Departments of Medicine [D. B. S., B. H., D. B. A., H. I. S., N. R.], Pathology [M. D., C. C.-C.], and Epidemiology and Biostatistics [D. V., G. H.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021

## Abstract

**Purpose:** Ansamycin antibiotics, including 17-allylamino-17-demethoxygeldanamycin (17-AAG), inhibit Hsp90 function and cause the selective degradation of signaling proteins that require this chaperone for folding. Because mutations in the androgen receptor (AR) and activation of HER2 and Akt may account, in part, for prostate cancer progression after castration or treatment with antiandrogens, we sought to determine whether an inhibitor of Hsp90 function could degrade these Hsp90 client proteins and inhibit the growth of prostate cancer xenografts with an acceptable therapeutic index.

**Experimental Design:** The effect of 17-AAG on the expression of Hsp90 regulated signaling proteins in prostate cancer cells and xenografts was determined. The pharmacodynamics of target protein degradation was associated with the toxicology and antitumor activity of the drug.

**Results:** 17-AAG caused the degradation of HER2, Akt, and both mutant and wild-type AR and the retinoblastoma-dependent G<sub>1</sub> growth arrest of prostate cancer cells. At nontoxic doses, 17-AAG caused a dose-dependent decline in AR, HER2, and Akt expression in prostate cancer xeno-

grafts. This decline was rapid, with a 97% loss of HER2 and an 80% loss of AR expression at 4 h. 17-AAG treatment at doses sufficient to induce AR, HER2, and Akt degradation resulted in the dose-dependent inhibition of androgen-dependent and -independent prostate cancer xenograft growth without toxicity.

**Conclusions:** These data demonstrate that, at a tolerable dose, inhibition of Hsp90 function by 17-AAG results in a marked reduction in HER2, AR, and Akt expression and inhibition of prostate tumor growth in mice. These results suggest that this drug may represent a new strategy for the treatment of prostate cancer.

## Introduction

Prostate cancer can be eradicated when localized, but systemic disease remains incurable. Androgen ablation is the standard treatment for advanced disease, but despite dramatic clinical responses, virtually all of the patients relapse (1). The mechanisms responsible for disease progression after castration or treatment with antiandrogens are complex and not fully understood. The AR<sup>2</sup> is expressed at normal or amplified levels in most patients with androgen-independent disease, and several gain of function mutations have been characterized (2-4). These include mutations within the ligand-binding domain that alter ligand-binding specificity and have been associated with clinical progression after antiandrogen therapy (4).

In a majority of cases, changes in the AR gene have not been identified suggesting that other mechanisms must be involved. Recent studies show that activation of receptor tyrosine kinase signaling pathways leads to phosphorylation of steroid receptors and their activation in a ligand-independent manner (5-8). In one experimental system, selection for prostate tumor cells that grow at low levels of androgen was associated with overexpression of the HER2 receptor tyrosine kinase (9). Thus, prostate cancer tumor growth after castration or treatment with hormone receptor antagonists may be mediated by AR mutation or its ligand-independent activation by upstream tyrosine kinase pathways.

Ansamycin antibiotics, exemplified by GM, are natural products that bind to a conserved pocket in the Hsp90 family of chaperone proteins (10-12). Hsp90 is not required for general cotranslational protein folding but does play a role in the refolding of proteins in cells exposed to stress (13, 14). It is also required for the conformational maturation of Raf and steroid receptors (15, 16). High concentrations of ansamycins prevent

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\* The abbreviations used are: AR, androgen receptor; GM, geldanamycin; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; RB, retinoblastoma; PI3k, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; EPL, egg-phospholipid; PSA, prostate specific antigen.

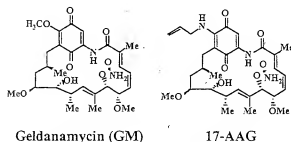


Fig. 1 Structures of GM and 17-AAG.

the binding of Hsp90 to its target proteins, which then cannot achieve their mature conformation (17). In addition, binding of ansamycins to the Hsp90-unfolded protein complex stabilizes the complex by preventing the ATP-dependent release of the chaperones (18, 19). The unfolded proteins in the complex are then ubiquitinated and targeted for degradation in the proteasome (20, 21).

Occupancy of the Hsp90 pocket by GM causes the degradation of several signaling proteins important in mediating prostate cancer growth. These include AR and members of the HER family of receptor tyrosine kinases (15, 18, 20–22). Here we show that treatment of prostate cancer cell lines with the GM derivative 17-AAG (Fig. 1) results in the degradation of HER2, and both wild-type and mutant AR, growth arrest, and an RB-dependent G<sub>1</sub> block. Furthermore, at nontoxic doses, 17-AAG induces the degradation of AR and HER family tyrosine kinases in prostate tumors, and inhibits their growth. These data suggest that 17-AAG is effective in inhibiting pathways required for the growth of advanced prostate cancer. Moreover, ansamycins can inhibit Hsp90 function in mice without toxicity and, thus, may represent a new strategy for the treatment of this disease.

## Materials and Methods

**Materials.** 17-AAG (NSC 330507) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). Drug was dissolved in DMSO to yield 10  $\mu$ M and 50 mg/ml stock solutions, and stored at  $-20^{\circ}\text{C}$ . The following antibodies were used: Akt (Cell Signaling, Beverly, MA; 1:500), AR (PharMingen, San Diego, CA; 1:250 for immunoblot and 1:100 for immunofluorescence), HER2 (Santa Cruz Biotechnology, Santa Cruz, CA; C-18, 1:1000), HER3 (Santa Cruz Biotechnology; C-17, 1:1000), Hsp70 (StressGene, Victoria, British Columbia, Canada; 1:1000), Hsp90 (StressGene; 1:1000), MAPK (Cell Signaling; 1:1000), p85 subunit of PI3k (Upstate Biotechnology, Lake Placid, NY; 1:2000), and RB (PharMingen; 1:1000).

**Cell Culture.** The human prostate cancer cell lines LNCaP, DU-145, and PC-3 were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 supplemented with 5–10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 50 units/ml of penicillin and streptomycin in a humidified 5% CO<sub>2</sub>/air atmosphere at 37°C.

LAPC-4 was generously provided by Charles Sawyers (UCLA, Los Angeles, CA) and maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and 10 nM R1881.

For the Alamar Blue proliferation assay,  $2-4 \times 10^5$  cells were plated in 96-well plates. Later (48 h), cells were treated with 17-AAG for 96 h or 0.01% DMSO as control. On day 4, Alamar Blue viability assay (AccuMed, Westlake, OH) was performed as described elsewhere (23). IC<sub>50</sub> and IC<sub>90</sub> were calculated as the doses of 17-AAG required to inhibit cell growth by 50 and 90%, respectively. Cell cycle distribution was assayed as described previously by Nusse *et al.* (24) with a Becton Dickinson fluorescence-activated cell sorter and analyzed by the Cell Cycle Multicycle system (Phoenix Flow System, San Diego, CA).

**Immunoblotting.** For immunoblotting, cells in culture were harvested in medium, washed twice in PBS, and then dissolved in SDS lysis buffer [50 mM Tris-HCl (pH 7.4) and 2% SDS], boiled for 10 min, and sonicated briefly. Cell lysates were cleared by centrifugation at 14,000  $\times$  g for 10 min, and supernatants were collected as the experimental samples. Lysates were added to sample buffer [0.125 M Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, and 77.5 mg/ml DTT], and equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked in 5% nonfat milk in Tris-buffered saline [0.1% Tween 20, 10 mM Tris (pH 7.4), and 150 mM NaCl] and subsequently probed with the antibody of interest. After incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were visualized by chemiluminescence (Amersham Corp., Piscataway, NJ). To prepare lysate from xenograft tumors, tumor tissue was homogenized in 2% SDS lysis buffer for 30 s then processed as above.

**Immunofluorescence.** For immunofluorescence,  $5 \times 10^5$  cells were plated onto fibronectin-coated chamber slides (Fisher Scientific). Cells were then incubated with 17-AAG, 500 nM, or 0.01% DMSO (control). At the indicated time points, slides were washed twice with ice-cold PBS, and fixed with methanol and acetone solution (1:1) for 5 min. Fixed monolayers were rehydrated with water and then blocked with 3% BSA in PBS solution. After nonspecific blocking, cells were incubated with anti-AR monoclonal antibody in 1% BSA in PBS at room temperature then washed three times with PBS. Monolayers were then incubated with an Alexa-488 conjugated secondary antibody for 1 h at room temperature. Nuclei were stained with 0.5  $\mu$ g/ml bis-benzamide (Hoechst 33342).

**Animal Studies.** Four- to six-week old *nu/nu* athymic male and female mice were obtained from the National Cancer Institute-Frederick Cancer Center (Frederick, MD) and maintained in ventilated caging. Experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol, and institutional guidelines for the proper and humane use of animals in research were followed. Before administration, 17-AAG was dissolved in an EPL vehicle developed for this purpose by the National Cancer Institute. To aid in the identification of an optimal dose and schedule, nontumor-bearing mice were treated by i.p. injection with 25–200 mg/kg of 17-AAG 5 days/week for 3 weeks or by the EPL vehicle alone. Serum samples were taken from each group, and equal volumes were pooled on days 5, 10, and 15 of treatment for serum

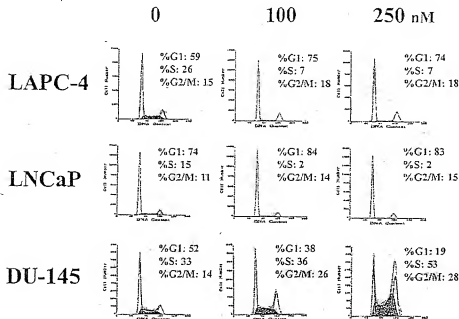


Fig. 2 17-AAG treatment of prostate cancer cell lines causes an RB-dependent cell cycle arrest. LAPC-4 (wild-type RB), LNCaP (wild-type RB), and DU-145 (mutant RB) cell lines were treated with 0 (0.01% DMSO), 100, or 250 nM 17-AAG for 48 h. Cell cycle distribution was assessed by fluorescence-activated cell sorter analysis. LAPC-4 and LNCaP cells arrested in G<sub>1</sub>. By immunofluorescent staining, DU-145 cells arrested in mitosis (DNS).

chemistry and liver function analysis. At sacrifice, plasma samples were collected for complete blood count. A gross necropsy was performed on all of the mice, and a complete necropsy, including histopathology, was performed on 1 animal/group.

Male animals were inoculated s.c. with minced tumor tissue from donor mice bearing the androgen-dependent CWR22 xenograft line. Females were inoculated in the same way with the androgen-independent xenografts CWR22R and CWRSA6. These variants were derived from tumors that regrew after castration-induced regression of CWR22 tumors (25). Tumor cells were injected together with reconstituted basement membrane (Matrigel; Collaborative Biotech, Bedford, MA). To maintain stable serum testosterone levels, 12.5-mg 90-day sustained release testosterone pellets (Innovative Research of America, Sarasota, FL) were placed s.c. before inoculation with androgen-dependent tumor. Tumor dimensions were measured twice a week with vernier calipers, and tumor volumes were calculated with the formula:  $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$ . Mice with established tumors 4–5 mm in diameter were selected for study ( $n = 5–8$  per treatment group). All of the mice received Augmentin (Amoxicillin/Clavulanate potassium; SmithKline Beecham) in their drinking water while on therapy. Mice were sacrificed by CO<sub>2</sub> euthanasia.

Both continuous and intermittent dosing schedules were studied. The “continuous” dosing schedule involved exposure to drug 5 days/week for 3 consecutive weeks. In the “intermittent” schedule, mice were treated with one 5-day cycle and then monitored for tumor progression. At progression, mice were treated with a second 5-day cycle of drug. In experiments with the androgen-dependent CWR22 tumor, serum PSA levels were measured with the PSA Assay kit (American Qualex Antibodies, San Clemente, CA).

In experiments designed to define the pharmacodynamic effects of 17-AAG on AR and HER-kinase expression, mice

with established tumors were treated with 17-AAG at the doses specified or with EPL alone. At the time of sacrifice, serum was collected, and tumors were flash frozen or fixed in 10% buffered formalin. Immunohistochemistry for AR (clone F39.4.1; 2 µg/ml; BioGenex, San Ramon, CA) and HER2 (Herceptest; DAKO Corp., Carpinteria, CA) were performed as described previously (25, 26). Serum concentrations of 17-AAG and 17-amino-17-demethoxygeldanamycin were determined by high-performance liquid chromatography based on the method of Egorin *et al.* (27).

**Statistical Analysis.** A permutation test was used to compare the average tumor volume over time between groups, using 5000 resamples. The null hypothesis of this test is that there is no difference in the change in tumor volumes over time between treatment groups. The statistic used to test this hypothesis was the sum of the squared differences between mean tumor volume summed over all time points, which in effect, compares the trajectories of the average tumor volume between treatment groups. It is defined as follows:

$$SS\_DEV = \sum_{i=1}^k (\bar{x}_i - \bar{y})^2,$$

where there are  $k$  time points and  $\bar{x}_i$  and  $\bar{y}$  are the average tumor volumes at time  $i$  in each treatment group. The Wilcoxon sum-rank test was used to compare treatment and control group serum PSA values.

## Results

**17-AAG Inhibited Prostate Cancer Cell Proliferation by Causing an RB-dependent G<sub>1</sub> Growth Arrest.** 17-AAG is a less toxic derivative of GM now in clinical trial. We found that

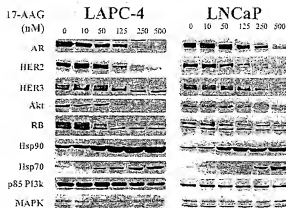


Fig. 3. Effect of 17-AAG on signaling proteins in LNCaP and LAPC-4 prostate cancer cells. Cells were treated for 24 h with 17-AAG at the indicated doses or DMSO (0.01%) as control. By immunoblot 17-AAG caused a dose-dependent decline in AR, HER2, HER3, and Akt expression and hypophosphorylation of RB. Levels of Hsp70 and Hsp90 were increased. No change in p85 PI3k or MAPK expression was noted.

17-AAG inhibited the anchorage-dependent growth of four representative prostate cancer cell lines. After 96 h of exposure,  $IC_{50}$ s ranged from 25–45 nM (LNCaP, 25 nM; LAPC-4, 40 nM; DU-145, 45 nM; and PC-3, 25 nM). At doses that caused complete growth arrest (75–125 nM for LNCaP and LAPC-4; and 150–200 nM for DU-145), prostate cell lines with intact RB (LNCaP, LAPC-4, and PC-3) arrested in G<sub>1</sub> after 17-AAG treatment, whereas DU-145, an androgen-independent cell line with mutated RB, arrested in mitosis (Fig. 2; data not shown). Growth arrest was accompanied by hypophosphorylation of RB (Fig. 3). These data are consistent with previous results in which we showed that the ansamycin Herblimycin A caused an RB-dependent G<sub>1</sub> block with RB-negative cells undergoing arrest in prometaphase (28, 29).

**17-AAG Caused the Down-Regulation of HER2, HER3, and AR.** Modulation of Hsp90 function by ansamycins causes the proteasomal degradation of a subset of cellular proteins. Most proteins and mRNAs are unchanged, as determined by evaluating multiple specific control proteins (PI3k and MAPK in Fig. 3 and DNS), the lack of change in the patterns of total cellular protein expression (DNS), and microarray analysis (30). HER2 is expressed at significant levels in both LAPC-4 and LNCaP, and is one of the most sensitive and rapidly degraded targets of 17-AAG (Fig. 3). HER3 and Akt levels also declined after 17-AAG treatment. 17-AAG increased the expression of the heat shock proteins Hsp90 and Hsp70.

Both wild-type AR (LAPC-4) and the Thr877Ala AR mutant found in LNCaP are sensitive to the drug as determined by immunoblot (Figs. 3 and 4). Exposure to 500 nM (24 h) of drug resulted in a 91% reduction in wild-type AR and a 92% reduction in the Thr877Ala AR mutant expressed by LNCaP cells. In LNCaP cells grown in serum-supplemented medium, most AR resides in the nucleus. Six and 12 h after 17-AAG treatment, nuclear AR staining was lost, and only faint cytoplasmic staining could be identified (Fig. 4). By 24 h, AR staining was again apparent with 52% of cells demonstrating detectable levels of nuclear AR by immunofluorescence. The intensity of nuclear AR staining within this population

was heterogeneous with only 10% of cell demonstrating nuclear AR staining equivalent to control levels (Fig. 4).

**Toxicology Studies.** The degradation of mutant AR and HER2 by 17-AAG suggests that this agent may be useful in the treatment of advanced prostate cancers. We sought to determine whether inhibition of these pathways could be accomplished *in vivo* with nontoxic doses of 17-AAG. The GM-Hsp90 domain has been studied in various species (yeast, bacteria, *Drosophila*, mouse, and human) and is highly conserved across species (11). Specifically, human and murine Hsp 90 $\alpha$  have >99% homology. Therefore, we evaluated the toxicity profile and pharmacology of 17-AAG in tumor and nontumor bearing *nu/nu* athymic mice. 17-AAG is metabolized by hepatic microsomes into at least five metabolites (27). The major metabolite, 17-A-G, is active and is equally potent in degrading HER2 and AR in LNCaP and LAPC-4 cell lines.<sup>6</sup> We measured 17-AAG and 17-A-G plasma concentrations after i.p. administration of one 50 mg/kg dose. 17-AAG was rapidly absorbed, and peak levels of >10  $\mu$ M were achieved within 30 min. Serum levels of 17-AAG >1  $\mu$ M were also detectable by high-performance liquid chromatography. Both 17-AAG and 17-A-G were then rapidly cleared with no detectable plasma levels 8 h after injection.

We found that the maximally tolerated dose of 17-AAG was schedule dependent and higher in control mice than in tumor-bearing mice. In nontumor bearing mice, treatment with three consecutive 5-day cycles of 75 mg/kg or more caused toxicity as evidenced by weight loss, elevated liver transaminase levels, anemia, and death (1 of 4 mice at the 75 mg/kg dose level, 3 of 4 mice at 125 mg/kg, and 4 of 4 mice at 200 mg/kg). Necropsy of mice after treatment with 17-AAG or the vehicle alone revealed peritonitis, possibly related to the i.p. route of administration, but no other gross or histological abnormalities. With less frequent dosing, up to 150 mg/kg/day of drug could be safely administered without evidence of toxicity (weight loss or death).

**17-AAG Caused a Reduction in AR, HER2, and HER3 Expression in Prostate Cancer Xenograft Tumors.** To determine whether nontoxic doses of 17-AAG could induce down-regulation of AR and HER kinases *in vivo*, we studied the effects of 17-AAG on the expression of these cellular proteins in the CWR22 xenograft model. In CWR22, the AR contains a mutation (histidine->tyrosine at residue 874) located within the ligand-binding pocket (31). Despite this mutation, androgens are still required for the growth of tumor growth (25). Several of these variants have been serially passaged, and two (CWR22R and CWRSA6) were selected for additional use.

We treated mice bearing CWR22 or CWRSA6 (androgen-independent) tumors with 25 or 50 mg/kg 17-AAG or EPL diluent for 4 days. After the final dose (8 h), the mice were sacrificed and the tumors removed. Four days of 17-AAG treatment resulted in a dose-dependent reduction in the expression of AR, HER2, HER3, and Akt (Fig. 5; CWR22 DNS). A dose of 50 mg/kg resulted in an 87% decline in AR, a 85% decline in HER2, a 50% decline in HER3, and a 60% decline in Akt

<sup>6</sup> F. Zheng, unpublished observations.

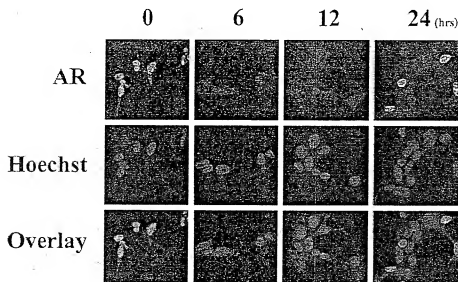


Fig. 4 Immunofluorescent staining of LNCaP cells for AR expression. Six and 12 h after treatment with 17-AAG (500 nm) LNCaP cells demonstrate a loss of nuclear AR expression. By 24 h a heterogeneous cell population is apparent with 52% of cells demonstrating at least partial recovery of AR staining.

expression in CWRSA6 tumors. Treatment was also associated with an 8-fold increase in Hsp70 and a 1.5-fold increase in Hsp90 levels. No change in the expression of PI3k was noted.

To characterize the kinetics of this effect, mice with well-established CWRSA6 tumors of comparable size were treated with a single dose of 17-AAG 50 mg/kg and sacrificed pretreatment and from 2 to 48 h afterward. A rapid, >50% decline in AR, HER2, and HER3 expression in the tumors was noted by 2 h (Fig. 5B). The maximal declines in AR and HER2 were noted at 4 h: a 97% reduction in HER2 and an 80% reduction in AR. By 24 h, AR expression returned to near baseline levels, whereas a rise in HER2 expression was not noted until 48 h after drug administration. The kinetics of HER2 recovery was similar in mice treated with 3 or 5 consecutive days of 17-AAG with its expression returning to near control levels 48 h after the final dose of therapy (DNS). With a single dose of therapy, the effect of 17-AAG on Akt expression was more delayed and less pronounced (maximum decline of 35% at 8 h) than that seen with AR, HER2, and HER3 (DNS).

In untreated CWRSA6 tumors, AR staining was compartmentalized in the nucleus (Fig. 5C). The frequency and intensity of this staining was diminished 4 and 8 h after 17-AAG administration. A steep decline in the mitotic index and a loss in membranous HER2 staining were also apparent at these time points. These data demonstrate that at nontoxic doses, 17-AAG induces the degradation of AR, HER2, HER3, and Akt in prostate tumors.

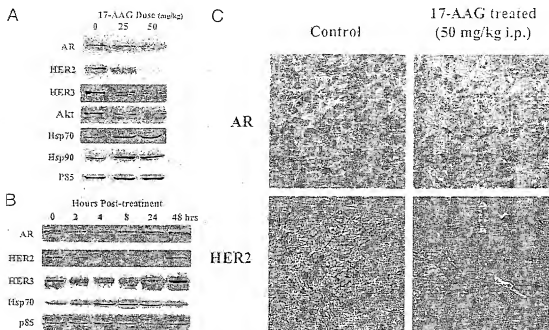
**17-AAG Inhibited the Growth of Androgen-dependent and Androgen-independent Prostate Cancers.** We studied the effects of two different dosing schedules of 17-AAG on the growth of CWR22, CWRSA6, and CWR22R tumors. The intermittent schedule consisted of a 5-day treatment cycle followed by a second cycle when definitive evidence of tumor regrowth occurred. The continuous schedule was comprised of three consecutive weekly 5-day cycles. Both regimens caused a dose-dependent delay in xenograft tumor growth in all three models (Table 1; Fig. 6). For example, with the continuous

schedule, 50 mg/kg 17-AAG caused 80% growth inhibition of CWRSA6 tumor growth when assessed on the day the controls required sacrifice [Table 1; Fig. 6A, mean tumor volume (treatment group) versus mean tumor volume (control) on day 29;  $P < 0.01$ ]. With the intermittent schedule, 17-AAG caused 87% growth inhibition of CWRSA6 tumor growth (Table 1; Fig. 6B;  $P < 0.01$ ). Similar results were noted with the parental CWR22 model and with a second androgen-independent subline CWR22R. Furthermore, in mice bearing CWR22 tumors, 17-AAG treatment (50 mg/kg dose level) was associated in an ~60% reduction in serum PSA (Day 25 serum PSA; intermittent schedule: 61% reduction versus control,  $P < 0.01$ ; continuous schedule: 62% reduction,  $P < 0.01$ ). These data demonstrate that at tolerable doses, 17-AAG inhibits the growth of prostate cancer cells *in vivo*. Additionally, growth inhibition correlates with a reduction in HER2, HER3, Akt, and the mutated AR (His874Tyr) expressed in the tumor.

## Discussion

In this report, we examined the effects of 17-AAG on prostate cancer growth using a panel of prostate cell lines and the CWR22 xenograft model. 17-AAG is a less-toxic derivative of the ansamycin GM and is now in clinical trial. We found that 17-AAG causes the selective degradation of a subset of proteins, many of them involved in mitogenic signaling. These included both wild-type (LAPC-4) and mutant (LNCaP, CWR22) ARs, and the HER2 and HER3 receptor tyrosine kinases. The expression of Akt, which is downstream of HER2 and which may modulate AR signaling in tumors with HER2 overexpression, is also down-regulated by the drug (8). Most cellular proteins including MAPK and PI3k were unaffected.

We have shown previously that exposure of cancer cells to the ansamycins GM and herbimycin A leads to a loss of cyclin D-associated kinase activity and an RB-dependent G<sub>1</sub> growth arrest (28). Cells lacking RB-function progress through G<sub>1</sub>



**Fig 5.** A, changes in target protein expression of CWRSA6 xenograft tumors from mice treated with 17-AAG at doses of 25 mg/kg and 50 mg/kg daily for 4 days. Control mice received vehicle only. Mice were sacrificed 8 h after the final treatment on day 4. Immunoblot demonstrates a dose-dependent decline in AR, HER2, HER3, and Akt expression. Levels of Hsp70 and Hsp90 were increased. No change in the control protein p85 PI3K was noted. This experiment was repeated with a second set of animals with equivalent results. B, mice with established CWRSA6 xenograft tumors treated with one dose of 17-AAG 50 mg/kg. Pretreatment and at the time points specified, mice were sacrificed and tumors removed. By immunoblot, a rapid decline in HER2, HER3, and AR expression is evident within 2 h of treatment. No change in p85 PI3K expression occurred. This experiment was repeated with a second set of animals with equivalent results. C, immunohistochemistry of CWRSA6 xenograft tumors demonstrating a loss of nuclear AR staining, a loss in membranous HER2 staining, and a reduction in the mitotic index 4 h after treatment with one dose of 17-AAG 50 mg/kg. Similar findings were also noted 8 h after treatment.

**Table 1.** Effect of continuous and intermittent dosing schedules of 17-AAG on the growth of CWR22, CWR22R and CWRSA6 xenografts.

Tumor	Dose (mg/kg)	Dosing regimen	Percent (%) Inhibition (day)	P
CWR22	25	Continuous	42 (24)	<0.01
	30	Continuous	67 (24)	<0.01
	30	Intermittent	51 (25)	0.01
CWRSA6	25	Continuous	56 (29)	0.07
	50	Continuous	80 (29)	<0.01
	50	Intermittent	87 (30)	<0.01
CWR22R	25	Continuous	49 (21)	0.01
	50	Continuous	68 (21)	0.02
	50	Intermittent	67 (22)	<0.01

normally in the presence of drug and arrest in prometaphase before undergoing apoptosis. D-cyclins are not direct targets of ansamycins, but their expression is controlled at the post-transcriptional level by a PI3K/Akt kinase-dependent pathway (32). Consistent with these findings, LNCaP and LAPC-4 (wild-type RB) cells arrested in G<sub>1</sub>, whereas DU-145, a cell line with mutant RB, arrested in mitosis.

We found that 17-AAG treatment reduced the expression of AR in prostate cancer xenografts and inhibited the growth of both

androgen-dependent and -independent tumors. The maximum effect of 17-AAG on AR expression in the tumors was evident 4–8 h after treatment with receptor levels returning to baseline by 24 h. The degradation of wild-type and mutant AR by drug suggests that this class of agents may be particularly effective in the treatment of advanced androgen-independent prostate cancer. The mechanisms responsible for the emergence of androgen-independent disease are complex and not fully understood. In a subset of patients treated with AR antagonists, clinical progression is associated with AR gene amplification or mutation (4, 33). AR amplification may result in sufficient AR pathway activation to allow for tumor growth at low levels of testosterone. Mutations in AR may convert antagonists into agonists or lead to constitutive ligand-independent activation of the receptor. For example the missense mutation (Thr877Ala) found in LNCaP cells has been identified in patients treated previously with flutamide, and this AR exhibits altered ligand specificity (4). Hydroxyflutamide, adrenal androgens, and estrogens induce its activation and promote cell growth. In some patients, tumor regression may occur after flutamide withdrawal (4, 34). These data suggest that mutation or overexpression of AR may play a role in the progression to an androgen-independent state. Although the antiproliferative effects of ansamycins may be multifactorial and the result of inhibition of AR-independent pathways, the ability of ansamycins to degrade mutant as well as wild-type

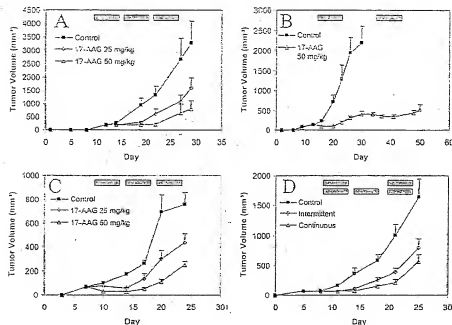


Fig 6 A and B, response of CWRSA6 androgen-independent xenografts to 17-AAG. C and D, response of CWR22 androgen-dependent xenografts to 17-AAG. Bars = SE.

forms of AR suggest that they may be useful in the treatment of such patients.

In most patients whose tumors relapse after hormonal therapy, mutations or amplification of the AR gene have not been identified. Several recent studies suggest that modulation of AR activity by growth factor activated tyrosine kinase pathways allows for prostate cancer growth at low androgen levels. The epidermal growth factor, insulin-like growth factor-1, keratinocyte growth factor, and interleukin 6 all induce AR phosphorylation and the expression of PSA, a downstream target of AR (6, 35). Several lines of evidence suggest that HER2 may be important in this process. HER2 overexpression and gene amplification have been identified in a subset of patients with prostate cancer (36, 37). HER2 overexpression is more common in patients treated previously with hormonal therapy and those with androgen-independent metastatic disease (37). CWR22 and LNCaP both express high levels of HER2, and Herceptin (Trastuzumab), a humanized monoclonal antibody that binds to HER2, inhibits the growth of these xenografts (26). A mechanism for this effect has been proposed by Yeh *et al.* (7) who demonstrated that HER2 activates AR by inducing its phosphorylation by MAPK. As 17-AAG targets both the AR and HER2 for degradation and thus inactivation, tumors that depend on tyrosine kinase pathway-mediated phosphorylation of AR for growth and survival after androgen ablation may be particularly sensitive to this agent.

Akt, which is downstream of HER2, also phosphorylates AR and may modulate AR signaling in tumors with HER2 overexpression (8). We found that 17-AAG down-regulated the expression of Akt in a dose-dependent manner in the xenograft tumors, although this effect was delayed and of a lesser degree than the effect of 17-AAG on AR and HER2. Hsp90 has been reported recently to associate with Akt and regulate its activity (38). Therefore, Akt may be a direct target of 17-AAG. Alternatively, the reduction in

Akt expression caused by 17-AAG could be secondary to down-regulation of other pathways by the drug.

In this report, we show that 17-AAG, an inhibitor of Hsp90 function, delays the growth of prostate tumors *in vivo* at nontoxic doses. The inhibition of growth correlated with reduced expression of AR and HER-family kinases in the tumor. The results suggest that ansamycins may represent a new strategy for the treatment of advanced prostate cancers that depend on AR mutations or activated tyrosine kinases for tumor progression. Human trials to evaluate the efficacy of 17-AAG in this setting are now in progress.

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# Enhancement of Paclitaxel-Mediated Cytotoxicity in Lung Cancer Cells by 17-Allylamino Geldanamycin: In Vitro and In Vivo Analysis

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**Background.** It has previously been demonstrated that 17-allylamino geldanamycin (17-AAG) enhances paclitaxel-mediated cytotoxicity and downregulates vascular endothelial factor expression in non-small cell lung cancer. This project was designed to evaluate the tumoricidal and antiangiogenic effects of 17-AAG and paclitaxel in H358 non-small cell lung cancer cells grown as xenografts in nude mice.

**Methods.** In vitro cytotoxic drug combination effects were evaluated by (4, 5-dimethylthiazolo-2-yl)-2, 5-diphenyl tetrazolium bromide-based proliferation assays. The combinations of 17-AAG and paclitaxel were administered intraperitoneally in nude mice bearing H358 tumor xenografts. Tumor volumes were measured weekly. Tumor expression of erbB2, vascular endothelial cell growth factor, von Willebrand factor (tumor microvasculature), and activated caspase 3 (apoptosis) were determined by immunohistochemistry.

**Results.** Five- to 22-fold enhancement of paclitaxel cytotoxicity was achieved by paclitaxel + 17-AAG combination that was paralleled with marked induction of apoptosis. This combination treatment profoundly sup-

pressed tumor growth and significantly prolonged survival of mice bearing H358 xenografts. Immunohistochemical staining of tumor tissues indicated profound reduction of vascular endothelial cell growth factor expression associated with reduction of microvasculature in tumors treated with 17-AAG. Apoptotic cells were more abundant in tumors treated with 17-AAG + paclitaxel than in those treated with 17-AAG or paclitaxel alone.

**Conclusions.** Concurrent exposure of H358 cells to 17-AAG and paclitaxel resulted in supraadditive growth inhibition effects in vitro and in vivo. Analysis of molecular markers of tumor tissues indicated that therapeutic drug levels could be achieved with this chemotherapy regimen leading to significant biological responses. Moreover, 17-AAG-mediated suppression of vascular endothelial cell growth factor production by tumor cells may contribute to the antitumor effects of this drug combination in vivo.

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Non-small cell lung cancers (NSCLC) frequently exhibit resistance to chemotherapy and ionizing radiation. Although dose-intensive regimens may increase response rates, they are frequently associated with severe systemic side effects. Treatment strategies designed to sensitize tumor cells to chemotherapeutic agents or radiation therapy may allow dose reduction, thereby diminishing systemic toxicity of cancer treatments.

The molecular basis of chemoresistance in cancer is complex and appears to involve both genetic and epigenetic factors, including overexpression of erbB1 and erbB2, encoding the epidermal growth factor receptor (EGFr) and the orphan receptor HER2/neu respectively. Inhibition of the function or the expression of the EGFr or

HER2/neu sensitizes tumor cells, including those of NSCLC histology, to standard cytotoxic agents such as cisplatin or paclitaxel [1-3]. Previously we demonstrated that sensitivity to paclitaxel could be enhanced in NSCLC cells overexpressing erbB2 after treatment with 17-allylamino geldanamycin (17-AAG) [4]. This agent has been selected for clinical development at the National Cancer Institute because of its activity against cell lines derived from a variety of human malignancies [5, 6]; antitumor effects of this compound relate in part to its ability to inhibit the expression of various oncoproteins including EGFr and HER2/neu at nanomolar to micromolar concentrations [6, 7].

Overexpression of EGFr or HER2/neu oncoproteins in lung, esophageal, breast, and ovarian cancers correlates with locally advanced disease, distant metastases, and diminished survival in patients with these malignancies [8-10]. In vitro experimental data indicate that tumor cells overexpressing these oncoproteins exhibit one or more phenotypes associated with local invasion or dis-

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tant metastasis in vivo, including downregulation of E-cadherin expression, increased expression of matrix metalloproteinases, and vascular endothelial growth factor (VEGF), as well as accelerated invasion through artificial extracellular matrix [11-13]. Conceivably, inhibition of erbB-mediated signal transduction by monoclonal antibodies, which antagonize receptor ligand binding, or compounds such as 17-AAG, which deplete erbB1/erbB2 expression, may effectively reduce the metastatic potential of cancer cells. Exploiting the latter approach, we have recently demonstrated that treatment of NSCLC cells with 17-AAG at nanomolar concentrations for 48 hours results in profound suppression of VEGF and matrix metalloproteinase-9 secretion, as well as inhibition of cell motility through the artificial extracellular matrix membrane Matrigel (Sigma, St. Louis, MO) [14]. The relevance of these findings, particularly the antiangiogenic effects of 17-AAG-mediated suppression of VEGF production, have not been defined.

In the present study, we sought to examine the in vivo effects of paclitaxel and 17-AAG using nude mice bearing H358 NSCLC xenografts. Herein, we demonstrate that 17-AAG + paclitaxel treatment mediates profound retardation of tumor growth, and significant prolongation of survival in tumor-bearing animals. The in vitro effects of 17-AAG on H358 cells were closely reproduced in vivo: diminished erbB2 and VEGF expression in xenografts correlated with reduced tumor capillary density in animals receiving treatments containing 17-AAG. Apoptosis was most pronounced in xenografts from animals treated with 17-AAG + paclitaxel. Collectively these data support further evaluation of 17-AAG in combination with paclitaxel in lung cancer patients.

## Material and Methods

### Cells and Reagents

The NSCLC cell line H358 was purchased from American Tissue Culture Collection (Manassas, VA). Cells were maintained in RPMI media supplemented with glutamine (1 mmol/L), streptomycin (100 µg/mL)/penicillin (100 U/mL), and 10% of fetal calf serum. Normal human bronchial epithelial cells were purchased from Clonetics Corp (Walkersville, MD) and maintained in bronchial epithelial cell basal media (Clonetics Corp). The 17-AAG, obtained from the Drug Synthesis & Chemistry Branch, Developmental Therapeutic Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, was dissolved in dimethylsulfoxide (DMSO) to yield a 100 µmol/L stock solution and stored at -70°C. The selective erbB2 tyrosine kinase inhibitor AG825 was obtained from Calbiochem/Oncogene Research Products (Cambridge, MA), dissolved in DMSO, and stored as 10 mmol/L stock solution. All experiments involving these two compounds were performed under subdued light conditions. Paclitaxel (Taxol, USP) was purchased from Bristol-Myers Squibb (Princeton, NJ). 4,5-Dimethylthiazolo-2-yl-2,5-diphenyl tetrazolium bromide was purchased from Sigma (St. Louis, MO). Recombinant human epidermal

growth factor (EGF) (R&D, Minneapolis, MN) and the anti-erbB1 and anti-erbB2 monoclonal antibodies (Calbiochem/Oncogene Research Products, Cambridge, MA) were constituted in phosphate-buffered saline and stored at 4°C as recommended by the manufacturers. Human VEGF enzyme-linked immunosorbent assay kit was obtained from R&D. A formulation of egg phospholipid emulsion in dextrose solution was used to dissolve 17-AAG for parenteral administration.

### Immunofluorescent Staining and Flow Cytometric Analysis of erbB1 and erbB2 Expression

Surface expression of erbB1 and erbB2 on H358 or normal human bronchial epithelial cells was quantitated by flow cytometry using a Beckton-Dickinson fluorescence-activated cell sorter as described previously [14].

### Quantitation of Vascular Endothelial Cell Growth Factor Production by H358 Cells

Cells were grown to 80% confluency in 12-well tissue culture plates, washed once with phosphate-buffered saline and media replenished with 1 mL of RPMI with 1% fetal calf serum with or without 20 ng/mL of EGF. In 17-AAG- or AG825-treated groups, appropriate aliquots of 17-AAG or AG825 stocks were added into the culture media to yield desired drug concentrations 2 hours before stimulating tumor cells with EGF. After 24 hours of incubation, conditioned media were harvested and frozen at -70°C. Cells from each well were collected and cellular protein was assayed by BCA technique (Pierce, Rockford, IL). The VEGF levels in the conditioned media were measured by enzyme-linked immunosorbent assay using a commercially available kit and expressed as picograms per milliliter per 24 hours per milligram of cellular protein.

### In Vitro Evaluation of Drug Cytotoxicity

Cells were seeded in flat-bottom 96-well microtiter plates (4,000 cells/well). After an overnight incubation, cells were treated with either paclitaxel alone or 17-AAG + paclitaxel combination. Cells were exposed to various concentrations (ranging from 4 to 1,000 nmol/L) of paclitaxel for 90 minutes followed by 96 hours of further incubation in normal media or media containing 17-AAG (20 or 40 nmol/L). At the end of the incubation period, viable cells were quantitated by (4, 5-dimethylthiazolo-2-yl)-2, 5-diphenyl tetrazolium bromide colorimetric assays as described by the manufacturer. Paclitaxel dose-response curves were plotted as a fraction of viable paclitaxel-treated cells relative to cells grown in normal media. The H358 cells treated with the 17-AAG + paclitaxel combination were plotted as fraction of viable cells relative to cells exposed to 17-AAG alone (to correct for the minor growth inhibitory effect of 17-AAG). Paclitaxel inhibitory concentration at 50% ( $IC_{50}$ ) values for cells treated with paclitaxel alone or paclitaxel in combination with 17-AAG were derived from respective dose-response curves. A reduction of paclitaxel  $IC_{50}$  values in cells treated with the drug combination indicated increased cellular responsiveness to paclitaxel cytotoxic

effects mediated by 17-AAG. To further confirm the synergistic cytotoxic drug interaction effect of paclitaxel and 17-AAG, the combination index at 50% growth inhibition level ( $CI_{50}$ ) was calculated [15]. The  $CI_{50}$  values less than 1, equal to 1, or more than 1 indicate synergistic, additive, or antagonistic cytotoxic drug interactions, respectively.

#### Apoptosis and Caspase 3 Activity

The H358 cells treated with paclitaxel alone (50 or 200 nmol/L) or paclitaxel with 17-AAG (20 nmol/L) were harvested at 48 and 60 hours after drug treatment for measurement of caspase 3 activity and apoptosis, respectively. Caspase 3 activity in cell lysates was measured by colorimetric assay (R&D). After normalization for total protein in the cell lysates, caspase 3 activity was expressed as fold increase over levels detected in untreated control cells. Apoptosis was quantitated by flow cytometry techniques using the Apo-BrdU kit (Pharmingen, San Diego, CA) and protocols contained therein.

#### In Vivo H358 Human Tumor Xenografts Model

The H358 human tumor xenografts were created in hind flanks of nude mice by inoculation of  $10^7$  cells suspended in 100  $\mu$ L of phosphate-buffered saline. Palpable tumors of 170 to 200 mm<sup>3</sup> appeared approximately 4 weeks after tumor cell injection. Tumor-bearing animals then received either paclitaxel (1 mg/kg dissolved in 100  $\mu$ L of phosphate-buffered saline once per week for 4 weeks), 17-AAG (10 or 25 mg/kg in 100  $\mu$ L of carrier solution at three daily injections per week for 4 weeks) or paclitaxel + 17-AAG combinations (first doses of 17-AAG were administered concurrently with paclitaxel) by intraperitoneal injections. Control animals received phospholipid-based drug carrier solution alone. Orthogonal diameters of tumors were measured weekly. Animals were euthanized when tumors reached maximally allowable volumes of 2,500 to 2,800 mm<sup>3</sup>. Tumor volumes were estimated using the following formula:  $V = 0.52 \times a \times b \times c$ , where  $a$ ,  $b$ ,  $c$  are orthogonal diameters.

Representative xenografts were harvested at the end of treatment for evaluation of cellular expression of VEGF, von Willebrand factor, erbB2, or activated caspase 3 using immunohistochemical techniques.

#### Data Analysis

Data were expressed as mean  $\pm$  standard deviation of at least three independent experiments. Paired  $t$  test, Student's  $t$  test, and one-way analysis of variance (with Bonferroni test for pairwise comparisons) were performed for statistical analysis using Prism 2.0 software package (Graphpad Software, Inc, San Diego, CA).

#### Results

##### Depletion of erbB1 and erbB2 Expression on H358 Cells by 17-AAG

The H358 cells expressed high levels of erbB2 (approximately threefold higher than the level detected on nor-

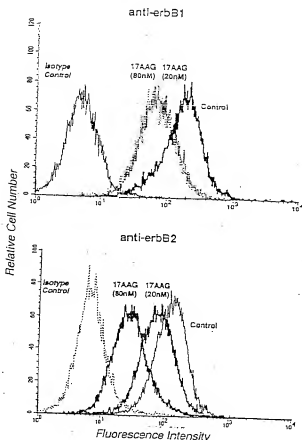


Fig 1. Immunofluorescence flow cytometric analysis of surface expression of erbB1 and erbB2 on H358 cells at baseline and after 24 hours of exposure to 17-allylamino geldanamycin (17-AAG) 20 or 80 nmol/L. Depletion of erbB1 (only at 80 nmol/L of 17-AAG) and erbB2 levels after 17-AAG treatment was indicated by significant reduction of erbB1 and erbB2 mean fluorescence intensity and shifting of the curves to the left. Representative data of three independent experiments that yielded similar results are shown.

mal human bronchial epithelial cells), with mean fluorescence intensity of  $160 \pm 11$ . Exposure of H358 cells to 20 or 80 nmol/L of 17-AAG for 24 hours resulted in a dose-dependent reduction of erbB2 mean fluorescence intensity to  $80 \pm 6$  and  $40 \pm 8$  ( $p < 0.01$  versus baseline controls,  $n = 3$ ) (Fig 1). Moreover, at the higher dose of 17-AAG, erbB2 expression was completely depleted in 65% of treated cells. In contrast, erbB1 expression in H358 cells was comparable to normal human bronchial epithelial cells, and depletion of erbB1 was achievable only at high concentration of 17-AAG. Exposure to 80 nmol/L of 17-AAG for 24 hours reduced erbB1 expression by 35% (mean fluorescence intensity of  $110 \pm 4$  in treated cells versus  $168 \pm 12$  in control cells,  $p < 0.01$ ); all tumor cells remained positively stained for this receptor (Fig 1). Similar to a previously published report [16], 24-hour exposure of tumor cells to 17-AAG was more efficient in depleting erbB2 than erbB1 membrane expression. The 17-AAG also significantly inhibited H358 cell growth in vitro with the estimated  $IC_{50}$  value of  $70 \pm 3$  nmol/L.

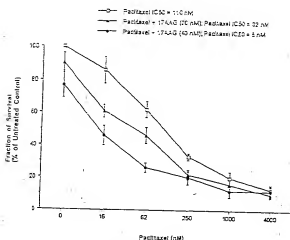


Fig 2. Enhancement of paclitaxel-mediated cytotoxicity in H358 cells by 17-allylamino geldanamycin (17-AAG) (20 or 40 nmol/L). The magnitude of growth inhibitory effect in cells treated with the drug combination was supraadditive, particularly at low doses of paclitaxel. Data are presented as mean  $\pm$  standard deviation of four independent experiments, paclitaxel inhibitory concentration at 50% (IC<sub>50</sub>) values are indicated in the legends.

#### Enhancement of Paclitaxel Cytotoxicity by 17-AAG In Vitro

Figure 2 demonstrates the dose-dependent growth inhibitory effects of paclitaxel or paclitaxel + 17-AAG combination in H358 cells. Paclitaxel effectively inhibited tumor cell viability (IC<sub>50</sub> = 110  $\pm$  8 nmol/L). Concurrent exposure of H358 cells to paclitaxel and 17-AAG resulted in a drastic reduction of paclitaxel IC<sub>50</sub> values (20  $\pm$  3 and 5  $\pm$

1 nmol/L at 20 nmol/L and 40 nmol/L of 17-AAG, respectively); these IC<sub>50</sub> values were significantly lower than the paclitaxel IC<sub>50</sub> value in cells treated with paclitaxel alone ( $p < 0.001$ ). The increased suppression of cell growth by the addition of 17-AAG to paclitaxel treatment (30% to 40% at lower spectrum of paclitaxel dose range) was higher than the mild 17-AAG-mediated growth inhibition. This finding suggested that 17-AAG enhanced the susceptibility of tumor cell to the cytotoxic effect of paclitaxel. Furthermore, the synergistic cytotoxic effect of this drug combination was confirmed by the combination index CI<sub>50</sub> values being significantly less than 1 (0.589  $\pm$  0.078 and 0.630  $\pm$  0.113 at 20 nmol/L and 40 nmol/L of 17-AAG, respectively,  $p < 0.01$ ). The 17-AAG did not activate caspase 3 or induce apoptosis in H358 cells; in contrast, caspase 3 activity was evident in H358 cells after treatment with 17-AAG + paclitaxel, which was higher than that observed after exposure to paclitaxel alone (Fig 3A). This was accompanied by a higher magnitude of induction of apoptosis (Fig 3B). For instance, 55%  $\pm$  8% and 60%  $\pm$  4% of apoptotic cells were detected after treatment with 50 nmol/L and 200 nmol/L of paclitaxel in conjunction with 17-AAG compared to 22%  $\pm$  3% and 28%  $\pm$  4% of apoptotic cells after exposure to paclitaxel alone ( $p < 0.01$ ).

#### Inhibition of Vascular Endothelial Cell Growth Factor Secretion From H358 Cells by 17-AAG and AG825 In Vitro

The H358 cells secreted high levels of VEGF into the culture media under basal conditions. Stimulation with EGF (20 ng/mL) resulted in a mild but significant elevation of VEGF (35,400  $\pm$  3,750 versus 27,596  $\pm$  2,500 pg/mL

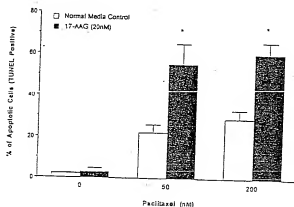
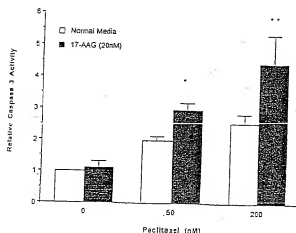


Fig 3. (A) Caspase 3 activity in H358 cells treated with paclitaxel (50 or 200 nmol/L) alone or in combination with 17-allylamino geldanamycin (17-AAG) (20 nmol/L). Significantly higher levels of caspase 3 activity was observed in H358 cells treated with 17-AAG and paclitaxel combination than those treated with paclitaxel alone. Caspase 3 activity is expressed as fold of increase over baseline levels detected in control cells. Data are presented as mean  $\pm$  standard deviation of four independent experiments, \* $p < 0.01$ , \*\* $p < 0.001$ . (B) Induction of apoptosis of H358 cells treated with paclitaxel (50 or 200 nmol/L) alone or in combination with 17-AAG (20 nmol/L). Apoptosis was quantitated in H358 cells harvested 60 hours after drug treatment by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay. Although 17-AAG at 20 nmol/L failed to induce apoptosis, combining 17-AAG with paclitaxel resulted in up to twofold increase in the induction of apoptosis. Data are presented as mean  $\pm$  standard deviation of four independent experiments, \* $p < 0.01$ .

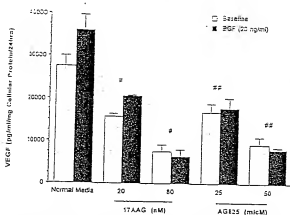


Fig 4. Levels of vascular endothelial cell growth factor (VEGF) in conditioned culture media of H358 cells at baseline or after stimulation with epidermal cell growth factor (EGF) (20 ng/mL) in the presence or absence of 17-allylamino geldanamycin (17-AAG) (20 or 80 nmol/L) or the selective erbB2 tyrosine kinase inhibitor AGS25 (25 or 50  $\mu$ mol/L). Not only did 17-AAG or AGS25 strongly inhibit vascular endothelial cell growth factor production by H358 cells at baseline conditions but they also effectively abrogated the upregulation of vascular endothelial cell growth factor production by epidermal cell growth factor. Data are presented as mean  $\pm$  standard deviation of four independent experiments. \* $p < 0.01$ , \*\* $p < 0.001$ .

per 24 hours per milligram of cellular protein,  $p < 0.01$ ). Treating H358 cells with 17-AAG (20 or 80 nmol/L) for 24 hours suppressed both basal as well as EGF-mediated upregulation of VEGF expression (Fig 4) in a dose-dependent manner with the magnitude of inhibition ranging from 40% to 80%.

Inhibition of EGF-mediated upregulation of VEGF production by H358 cells after 17-AAG exposure may result from its effect on downregulating both erbB1 and erbB2

expression. The erbB2 receptor, although lacking a cognate ligand, modulates signaling from other members of the erbB superfamily [17]. To delineate further the role of erbB2 in EGF-mediated upregulation of VEGF production, additional experiments were performed using the selective erbB2 tyrosine kinase antagonist AGS25 [18]. Treating H358 cells with AGS25 (25 or 50  $\mu$ mol/L) suppressed basal as well as EGF-mediated induction of VEGF secretion, to the same degree as observed after 17-AAG treatment (Fig 4).

#### In Vivo Tumoricidal Effect of Paclitaxel and 17-AAG

The in vivo experiment was designed to validate the in vitro findings of the synergistic cytotoxic effect and the potential antiangiogenic property of this combination (Fig 5). Parental administration of 17-AAG alone resulted in dose-dependent growth inhibition of H358 xenografts. Statistically significant reduction of tumor volumes and corresponding survival extensions were observed only in animals treated with the high dose of 17-AAG (25 mg/kg) (Fig 5 and Table 1). Significant inhibition of tumor growth and prolonged survivals were also noted in mice treated with paclitaxel (1 mg/kg) alone. More importantly, concurrent administration of 17-AAG (either at 10 or 25 mg/kg) and paclitaxel (1 mg/kg) resulted in a profound dose-dependent tumoricidal effects with 70% to 85% inhibition of tumor growth, and extension of survival from 3.5 months (low dose of 17-AAG with paclitaxel) to nearly 5 months (17-AAG 25 mg/kg and paclitaxel), representing a two- to threefold prolongation of life expectancy of tumor-bearing animals (Fig 5 and Table 1). The tumor volumes measured at 8 weeks after the beginning of combination chemotherapy were significantly less than those predicted by assuming additive drug effects (Table 1). Furthermore, the mean survival extensions of animals treated with 17-AAG + paclitaxel com-

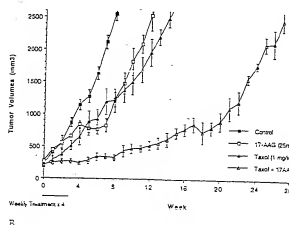
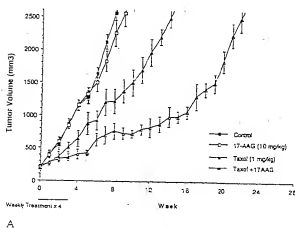


Fig 5. Enhancement of paclitaxel tumoricidal effect by 17-allylamino geldanamycin (17-AAG) in nude mice bearing H358 tumor xenografts. Animals were treated with either 10 mg/kg of 17-AAG (A) or 25 mg/kg of 17-AAG (B) in combination with paclitaxel (1 mg/kg) weekly for 4 weeks by intraperitoneal injections. Treatment schedule consisted of paclitaxel one injection/wk or 17-AAG three daily consecutive injections/wk for 4 weeks. Controls were animals treated with mock injection of the phospholipid-based carrier solution, with 17-AAG alone or with paclitaxel alone. Tumor dimensions were measured weekly until sacrifice. Data are presented as mean  $\pm$  standard deviation,  $n = 8$  per group. (Taxol = paclitaxel.)

Table 1. *In Vivo* Tumoricidal Effects of 17-AAG, Paclitaxel, and 17-AAG + Paclitaxel Combination

Group	Survival <sup>a</sup> (wk)	Mean Extension of Survival (wk)	Tumor Volumes at 8 Weeks (mm <sup>3</sup> )
Control	7.8 ± 0.8	N/A	2,557 ± 110
17-AAG (10 mg/kg)	9.2 ± 1.6	1.4	2,370 ± 300
17-AAG (25 mg/kg)	13.5 ± 0.5 <sup>b</sup>	5.7	1,077 ± 56
Paclitaxel (1 mg/kg)	14.3 ± 1.6 <sup>b</sup>	6.5	1,210 ± 183
17-AAG (10) + paclitaxel	22.6 ± 1.6 <sup>b</sup>	14.8	762 ± 120 (1174) <sup>c</sup>
17-AAG (25) + paclitaxel	26.8 ± 3.2 <sup>b</sup>	19.0	328 ± 45 (508) <sup>c</sup>

Mean ± SD (n = 8 per group).

<sup>a</sup>Time elapsed from the onset of therapy to reaching tumor volumes of 2,500 mm<sup>3</sup>. <sup>b</sup>p < 0.001 versus control by analysis of variance and Bonferroni test. <sup>c</sup>Predicted mean tumor volumes by assuming additive drug effects.

17-AAG = 17-allylamino geldanamycin.

binations were longer than the algebraic sums of the extensions resulting from each treatment alone (Table 1). Collectively, these data suggested *in vivo* synergistic tumoricidal drug effects.

Immunohistochemical analysis of erbB2 and VEGF expression, capillary density, and apoptosis in tumor nodules harvested 24 hours after completion of chemotherapy is shown in Figure 6. A profound reduction of membrane-bound erbB2 expression was observed in tumors from mice treated with either 17-AAG alone or 17-AAG + paclitaxel, suggesting that therapeutic levels of 17-AAG were achieved in xenografts. In parallel with *in vitro* observation of 17-AAG-mediated inhibition of VEGF production by H358 cells, cytoplasmic VEGF expression was also significantly decreased in tumors harvested from mice treated with 17-AAG or 17-AAG + paclitaxel (Table 2). Interestingly, mild-to-moderate reduction of cytoplasmic VEGF expression, was also noted

in tumors treated with paclitaxel alone, similar to previously published observations [19]. Capillary density of tumor nodules was evaluated by staining for von Willebrand factor, which is expressed by capillary endothelial cells. Diffuse positive endothelial staining observed in control tumors was slightly diminished in tumors treated with paclitaxel. von Willebrand factor expression was markedly reduced in tumor treated with 17-AAG or 17-AAG + paclitaxel, indicating that reduction of VEGF expression coincided with diminished tumor angiogenesis (Table 2). Abundance of activated caspase 3-positive cells (indicative of ongoing apoptosis, dark brown staining cells) could be observed in tumors treated with the 17-AAG + paclitaxel drug combinations (Fig 6). Apoptotic cells were less frequently noted in paclitaxel-treated tumors, and rarely were they seen in control or 17-AAG-treated tumors.

## Comment

Previous studies have demonstrated that members of the erbB superfamily (particularly erbB1 and erbB2, which are frequently overexpressed in NSCLC cells) play important roles in mediating the response of tumor cells to cytotoxic stress and modulating their susceptibility to chemotherapeutic agents. Overexpression of erbB2 in cancer cells enhances resistance to various cytotoxic agents including cisplatin and paclitaxel as well as irradiation. Upregulation of DNA repair mechanisms mediated by erbB2 overexpression contributes to chemotherapy and radiation resistance in cancer cells [3]. In contrast, upregulation of the cyclin-dependent kinase inhibitor p21, which inhibits progression of cells into the M phase of the cell cycle, is a major mechanism of paclitaxel resistance in cancer cells overexpressing erbB2 [20, 21]. Overexpression of erbB1 or activation of erbB1 receptor tyrosine kinase activity by EGF similarly renders tumor cells refractory to paclitaxel cytotoxicity [22]. Inhibition of the function or phenotypic expression of erbB1 or erbB2 by monoclonal antibodies or low molecular weight compounds abrogates chemoresistance mediated by erbB overexpression in cancer cells.

We have been interested in developing strategies to enhance the cytotoxic effects of conventional chemotherapeutic agents such as paclitaxel in lung or esophageal cancers by exploiting the current understanding of the relationship between the erbB signal transduction pathways and paclitaxel sensitivity. By targeting the erbB oncogenes using low molecular weight compounds such as 17-AAG we may avoid technical issues that may limit the efficacy of anti-erbB monoclonal antibodies, antisense constructs, or intracellular anti-HER2/neu single chain antibodies. Previously, we have demonstrated sequence-dependent enhancement of paclitaxel cytotoxicity in NSCLC by this compound [4]. Concurrent but not sequential 17-AAG/paclitaxel drug exposure significantly sensitized NSCLC cells expressing high levels of erbB2 to paclitaxel. This salutary effect has been extended to esophageal cancer cells expressing elevated levels of erbB2 (unpublished data). The *in vivo* experiments dem-

Table 2. *Semiquantitative Analysis of Immunohistochemical Staining of Tumor Tissues for Molecular Markers (n = 3)*

Group	erbB2	VEGF	vW Factor	Activated Caspase 3
Control	++++	+++	+++	0
17-AAG (10 mg/kg)	++	++	++	0
17-AAG (25 mg/kg)	+	+	++	0
Paclitaxel (1 mg/kg)	+++	++	++	+
17-AAG (10) + paclitaxel	++	++	++	++
17-AAG (25) + paclitaxel	+	+	+	+++

17-AAG = 17-allylamino geldanamycin; VEGF = vascular endothelial cell growth factor; vW = von Willebrand.

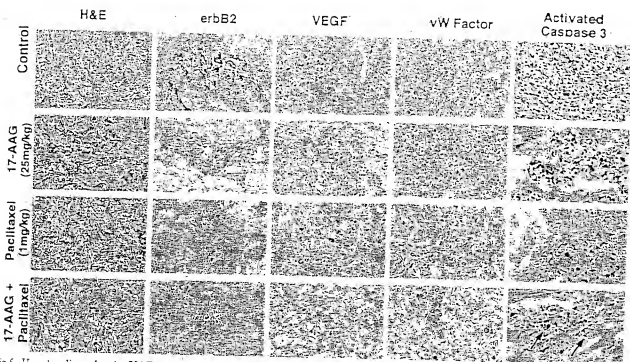


Fig 8. Hematoxylin and eosin (H&E) and immunohistochemical staining of tumor tissues harvested 24 hours after completion of the full 4-week course chemotherapy for molecular markers including erbB2, vascular endothelial cell growth factor (VEGF), von Willebrand (vW) factor (staining for endothelial cells to evaluate tumor microvasculature) and activated caspase 3 (only present in apoptotic cells). Representative photomicrographs of tumors treated with phosphate-buffered saline (control), 17-allylamino geldanamycin (17-AAG) (25 mg/kg), paclitaxel (1 mg/kg), or both are shown here. Hematoxylin and eosin: magnification,  $\times 200$ ; immunohistochemical staining: magnification,  $\times 400$ .

onstrating profound tumoricidal effects of the paclitaxel and 17-AAG combination extended in vitro observations of synergistic cytotoxic drug interaction of these two agents. However, it is difficult to prove synergism of drug interactions in vivo. The observations that tumor volumes in animals treated by the drug combinations were smaller than predicted by assuming additive antitumor drug effects (Table 1) and the survivals of tumor-bearing animals treated with the drug combination were longer than the algebraic sums of the extension of survivals achieved by individual drug treatment alone strongly support the notion of potentiation of paclitaxel tumoricidal effect by 17-AAG.

In addition to being a chemotherapy sensitizer, our studies have shown that 17-AAG is a powerful inhibitor of VEGF production by tumor cells through its negative effect on erbB signal transduction pathways, which are known to transcriptionally regulate VEGF gene expression [13, 23]. Significant information was derived from the molecular analysis of tumor samples harvested after completion of a 4-week course of chemotherapy. This time point was chosen to clearly demonstrate the antiangiogenic effect of 17-AAG. Whereas depletion of VEGF secretion by H358 cells occurs within hours of 17-AAG exposure, alteration of tumor microvasculature in response to reduced VEGF levels would take more time to develop. The in vivo effects of 17-AAG on erbB2 or VEGF expression and the induction of apoptosis by paclitaxel

(with or without 17-AAG) were probably derived from the last treatment rather than a cumulative effect of the four cycles. Almost complete depletion of erbB2 expression in tumors treated with regimens containing 17-AAG provided direct proof that therapeutic drug levels were achieved using the treatment schedule discussed. In conjunction with depletion of erbB2 expression, significant reduction of VEGF cytoplasmic expression in H358 tumor cells and inhibition of angiogenesis were also observed. These data clearly demonstrate the biological significance of 17-AAG-mediated inhibition of VEGF production, and affirm our in vitro experiments that suggested that 17-AAG exhibits antiangiogenic effects. In this context, 17-AAG played the dual role of a paclitaxel-sensitizing and an antiangiogenic agent. This drug combination strategy appears quite analogous to recent anticancer regimens combining a cytotoxic agent and an antiangiogenic agent (either an anti-VEGF antibody or a VEGF receptor pharmacologic antagonist) [24, 25]. The individual contribution of the paclitaxel sensitization and the antiangiogenic properties of 17-AAG are difficult to define at this time.

The phase I clinical trial using 17-AAG (daily 1-hour intravenous infusion for 5 days every 3 weeks) as a single agent to treat advanced solid malignancies is in progress at the National Cancer Institute, and initial data indicate that hepatotoxicity is the dose-limiting factor as predicted from preclinical studies (Jenn Grem and Richard



Wilson, National Cancer Institute, Bethesda, MD, personal communication). The peak plasma drug levels at maximally tolerated dose were 1 to 2  $\mu\text{mol/L}$ , which are well above the concentrations required to achieve synergistic drug interaction in vitro. The acceptable drug toxicity profile derived from the phase I trial together with the powerful tumoricidal effect of this drug combination observed in our present studies support evaluation of 17-AAG in combination with paclitaxel in patients with NSCLC and esophageal cancers exhibiting high level HER2/neu expression.

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## DISCUSSION

DR HARVEY I. PASS (Detroit, MI): Very nice, Dr Nguyen.

I have a couple of questions. The relationship between erbB-2 and this whole situation, what is the evidence that AAG is actually working selectively through an erbB-2 mechanism and is not having other effects, and have you done experiments to block either with antibody or making it even better using Herceptin (Genentech, San Francisco, CA)?

The second question is, this could also be explained by overcoming resistance to paclitaxel. Do you have any data with regard to your cell line with regard to  $\beta$  tubulin mutations in this cell line?

DR NGUYEN: To answer your first question, in terms of the impact of 17-AAG on erbB-2-mediated signal transduction path-

way, you are perfectly right. This compound not only targets erbB-2, it also targets other signal transduction pathways as well as other oncoproteins. To specifically target erbB-2 pathway, we used Herceptin and we also used AG825, which is a selective erbB-2 antagonist. We observed similar enhancement of paclitaxel-mediated cytotoxicity in NSChC cells by AG825. Herceptin, on the other hand, we did not see such a powerful sensitization effect, and the reason for that is unclear at this point.

What is your second question, Dr Pass?

DR PASS: The question of whether this is simply an overcoming chemoresistance phenomenon. Do you have any data whether AAG has any effect on cell lines with  $\beta$  tubulin mutations, and does your cell line have  $\beta$  tubulin mutation?

DR NGUYEN: No, we have not done that. We recently looked at the mechanisms of this combination effect, and it is related to the ability of 17-AAG to suppress p21, and that allowed the cells to traverse the G2M check point and accumulate the cells in mitosis, and p21 actually inhibits cdC2/p54 kinase, which is known to play an important role in paclitaxel-mediated apoptosis. So we observed that by using this combination the p21 levels decreased, associated with higher fraction of cells in mitosis and augmentation of paclitaxel-mediated apoptosis.

DR PASS: Do you have any data with any other chemotoxics?

DR NGUYEN: It has been looked at before with 17-AAG-sensitized tumor cells to cisplatin and the other chemotherapeutics.

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## Notice From the American Board of Thoracic Surgery

The 2001 Part I (written) examination will be held at the Hotel Sofiel, 5500 North River Rd, Rosemont, IL 60018-5194, on November 18, 2001. The closing date for registration was August 1, 2001.

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# Modulation of Metastasis Phenotypes of Non-Small Cell Lung Cancer Cells by 17-Allylamino 17-Demethoxy Geldanamycin

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**Background.** Cancer cells that overexpress c-erbB oncogenes exhibit resistance to chemotherapy, enhanced tumorigenicity, as well as increased propensity for metastasis. The aim of this study was to investigate if depletion of erbB-1/EGFR and erbB-2/HER2neu oncogene products by 17-allylamino 17-demethoxy Geldanamycin (17AAGA) could diminish the metastatic potential of non-small cell lung cancer (NSCLC) cells that express varying levels of the erbB1/erbB2 oncogenes.

**Methods.** NSCLC cell lines (H460, H358, H322, or H661) were assayed for expression of erbB1 and erbB2, the cell adhesion molecule E-cadherin, secretion of the matrix metalloproteinase 9 (MMP-9), and vascular endothelial cell growth factor (VEGF), as well as their ability to invade Matrigel after 48-hour exposure to 17AAGA.

**Results.** 17AAGA significantly depleted erbB1 or erbB2 levels in NSCLC cells expressing high levels of

these proteins, and effectively inhibited their growth with  $IC_{50}$  values ranging from 50 to 90 nmol/L. Moreover, drug treatment enhanced E-cadherin expression in H322 and H358 cells, and inhibited secretion of MMP-9 and VEGF secretion by tumor cells. 17AAGA diminished hypoxia-induced upregulation of VEGF expression as well as growth factor-mediated augmentation of MMP-9 secretion, and profoundly inhibited the ability of H322 and H358 cells to migrate through Matrigel in response to chemoattractants.

**Conclusions.** In addition to its known antiproliferative and chemosensitization effects, 17AAGA inhibits the metastatic phenotype of lung cancer cells. 17AAGA may be a novel pharmacologic agent for specific molecular intervention in lung cancer patients.

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Patients with non-small cell lung cancer (NSCLC) frequently present with either locally advanced (stage IIIA/B) or systemic disease (stage IV). The overall prognosis for these individuals is very poor, with the median survival less than 9 to 12 months despite an aggressive combination of chemo and radiotherapy. More importantly, the main mode of treatment failure after curative-intent therapy for early-stage NSCLC is systemic metastasis [1]. Acknowledging the fact that mature data of phase II/III clinical trials addressing the values of adjuvant chemotherapy for completely resected early-stage NSCLC are still forthcoming, there is no evidence that adjuvant therapy has any impact on the outcome of surgically treated NSCLC [2]. Micrometastases derived from lung cancers may have low proliferative activity, rendering them insensitive to standard cytotoxic agents. Hence, there is an urgent need for novel antineo-

plastic agents that exhibit strong antiproliferative effects as well as a capacity to inhibit the metastatic potential of tumor cells.

Solid tumor metastases arise by a multistep process regulated by complex interactions between tumor cells and adjacent stromal tissues [3]. In association with altered expression of adhesion molecules such as E-cadherin, cells detach from the main tumor mass and migrate through the extracellular matrix, degraded by tumor-derived matrix metalloproteinases (MMPs). After dissemination, cells exit from the vasculature and establish metastatic deposits, facilitated by the expression of integrin or hyaluronate (CD44) receptors, and the secretion of angiogenesis factors such as vascular endothelial cell growth factor (VEGF).

Experimental and clinical data have indicated that overexpression of erbB1 and erbB2 protooncogenes encoding the epidermal growth factor receptor (EGFR) and the orphan receptor (HER2/neu), respectively, correlates with locally advanced disease, distant metastases, and diminished survival in patients with breast, lung, esophageal, and ovarian carcinomas [4-7]. Tumor cells overexpressing these protooncogenes exhibit one or more phenotypes correlating with enhanced metastatic potential

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including downregulation of E-cadherin expression, increased secretion of MMP-9 and VEGF, and accelerated invasion through extracellular matrix [8-10]. Therapeutic strategies that specifically target erbB should inhibit mitogenic signaling via erbB1 and erbB2 pathways and may reduce the proliferation and metastatic potential of cancer cells; indeed, treatment of cells overexpressing the erbB1 or erbB2 gene products with antagonistic monoclonal antibodies significantly inhibits their metastatic phenotype [10, 11].

The benzoquinone ansamycin antibiotic geldanamycin (GA) and its less toxic synthetic derivative, 17-allylamino 17-demethoxygeldanamycin (17AAGA), have been recently selected for clinical development at the National Cancer Institute based upon their activity against cell lines derived from a variety of human malignancies [12, 13]. The growth inhibitory effects of geldanamycin and 17AAGA appear related to their ability to inhibit the expression of several cellular oncoproteins, including erbB1 and erbB2 [13]. In the present study, we sought to evaluate if 17AAGA could modulate the metastatic potential of lung cancer cells overexpressing the erbB1 and erbB2 gene products. Herein, we demonstrate that treatment of NSCLC cells with 17AAGA results in significant reduction of erbB1 or erbB2 expression, profound inhibition of cell proliferation, upregulation of E-cadherin expression, and downregulation of VEGF and MMP-9 secretion, paralleling with diminished capacity to invade extracellular matrix *in vitro*.

## Material and Methods

### Cells and Reagents

The NSCLC cells H460, H358, H661, and H322 were purchased from ATCC (Manassas, VA), and were grown in RPMI-1640 medium supplemented with glutamine (1 mmol/L), penicillin (100 U/mL)/streptomycin (100 µg/mL), and 10% fetal bovine serum (FBS) (all purchased from Biofluids, Rockville, MD). Normal human bronchial epithelial (NHBE) cells were purchased from Clonetics, Inc (Walkersville, MD) and maintained in bronchial epithelial cell basal media (Clonetics, Inc). 17AAGA was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, and dissolved in dimethyl sulfoxide to yield a 100 µmol/L stock solution, which was stored at -70°C. All experiments using this compound were performed under subdued lighting conditions. Recombinant human epidermal growth factor (EGF) and heregulin-α (HRG) (purchased from R&D, Minneapolis, MN), and the anti-erbB1 and anti-erbB2 monoclonal antibodies (purchased from Calbiochem/Oncogene Research Products, Cambridge, MA), were constituted in phosphate-buffered saline (PBS), and stored at 4°C as recommended by the manufacturers. The E-cadherin antibody was obtained from ICN Biomedicals, Inc (Aurora, OH).

### Immunofluorescent Staining and Flow Cytometric Analysis of erbB1, erbB2, and E-cadherin

Expression of erbB1, erbB2, and E-cadherin in NSCLC cell lines was quantitated by flow cytometry using a Becton-Dickinson (San Jose, CA) fluorescence-activated cell sorter (FACS). In brief, cells grown in either normal media with or without 17AAGA were harvested by trypsin/EDTA. To prevent proteolysis of surface adhesion molecules, cells harvested for E-cadherin staining were treated with 0.01% crystallized trypsin in the presence of 1 mmol/L of calcium, then washed in Ca/Mg-free PBS. Single-cell suspensions were incubated with either anti-erbB1, anti-erbB2, or anti-E-cadherin monoclonal antibodies for 60 minutes at room temperature. Cells were then washed with PBS, and incubated with fluorescent isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody at room temperature for 60 minutes in the dark. An irrelevant mouse IgG isotype monoclonal antibody was used as a negative control for FACS analysis. A minimum of  $10^4$  cells were analyzed by FACS flow cytometry. The magnitude of surface expression of these proteins was indicated by the mean fluorescence intensity (MFI) of positively stained cells. The MFI of isotype IgG control samples was always less than 10.

### In Situ Immunofluorescent Staining of E-cadherin

Cells were cultured in chamber slides (Nalge Nunc International Corp, Naperville, IL) until 80% confluent, washed with PBS, fixed with 1% paraformaldehyde for 10 minutes, and permeabilized with acetone for 5 minutes. Immunofluorescent staining for E-cadherin was performed similar to that described for flow cytometry. Slides were examined under the fluorescence microscope. Fluorescent photomicrographs were taken at 200× magnification.

### MMP-9 Assay

Cells were grown to confluence in six-well plates, washed twice with PBS, and then incubated with 2 mL of fresh serum-free RPMI-1640 medium. In the 17AAGA treatment group, cells were replenished with media containing either 20 or 80 nmol/L of 17AAGA. After 48 hours of incubation, conditioned media were harvested and frozen at -70°C until assayed for MMP-9 expression using an enzyme-linked immunosorbent assay (ELISA) kit (Calbiochem/Oncogene Research Products, Cambridge, MA). Cells from each well were collected, and total cellular protein was assayed by bicinchoninic acid (BCA) technique (Pierce, Rockford, IL). MMP-9 levels in the conditioned media were expressed as pg/mL/24 h/mg of total cellular protein.

### VEGF Assay

Cells were grown to 80% confluence in 24-well plates, washed once with PBS, and replenished with 2 mL of fresh RPMI-1640 alone or containing either 50 µmol/L or 100 µmol/L Cobalt Chloride (CoCl<sub>2</sub>) to simulate hypoxia [14]. In the 17AAGA treatment groups, appropriate aliquots of 100× 17AAGA stock were added to the culture

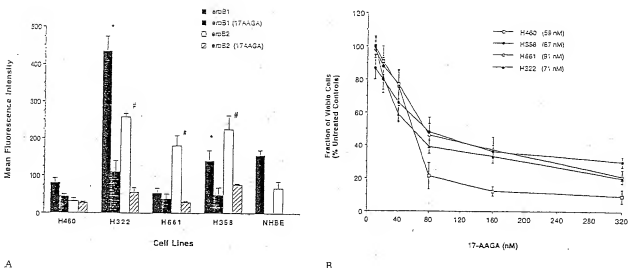


Fig 1. (A) Flow cytometric analysis of basal expression and 17AAGA-mediated depletion of erbB1 and erbB2 surface receptors in 4 NSCLC cell lines and normal human epithelial cells (NHBE). Exposure of these cells to 80 nmol/L of 17AAGA for 48 hours resulted in significant reduction of surface expression of these two receptors. Data are expressed as means  $\pm$  SD of three independent experiments (\* $p$  < 0.0001, # $p$  < 0.001). (B) Dose-dependent growth inhibition of NSCLC cells in vitro by 17AAGA. Cells were seeded in 96-well plates and, after an overnight incubation, were continuously exposed to varying doses of 17AAGA for 96 hours. Viable cells were quantitated by MTT assay. IC<sub>50</sub> values, estimated from these dose-response curves, are indicated in parentheses. Data are expressed as means  $\pm$  SD of three independent experiments.

media to yield a final concentration of 80 nmol/L. After 48-hour incubation, conditioned media were harvested and frozen at  $-70^{\circ}\text{C}$  until assayed for VEGF. VEGF levels in the conditioned media were evaluated using an ELISA kit (R&D) and expressed as ng/mL/24 h/10<sup>6</sup> cells.

#### Matrigel Invasion Assay

The chemoinvasion assay was performed as previously described [15]. Briefly, polyvinylpyrrolidone-free polycarbonate filters (10- $\mu\text{m}$  pore size; Neuroprobe, Gaithersburg, MD) coated with 500  $\mu\text{g/mL}$  of Egnlebreth-Holm-Swarm murine sarcoma basement membrane extract (Matrigel; Sigma Chemical Co, St. Louis, MO) were placed in a modified Boyden chamber (Neuroprobe, Gaithersburg, MD). Matrigel was diluted to the desired final concentration using cold ( $4^{\circ}\text{C}$ ) serum-free RPMI-1640 media. Filters were then placed in 15 mL of 500- $\mu\text{g/mL}$  Matrigel in 100  $\times$  15-mm Petri dishes and rotated at 45 rpm overnight at  $4^{\circ}\text{C}$ . Filters were air dried under a sterile hood immediately before use. Cells ( $6 \times 10^5/\text{mL}$ ) were suspended in serum-free RPMI and added to the upper chamber (30,000 cells/well). The lower chamber contained serum-free conditioned media from cultures of NIH 3T3 cells as a chemoattractant. Chambers were incubated at  $37^{\circ}\text{C}$  in 5% CO<sub>2</sub> for 6 hours. At the end of the incubation, cells on the upper surface of the filter were aspirated off and the filters were fixed in methanol and stained with Diff-Quik II, a Wright-Geimsa stain (Baxter, McGaw Park, IL). Cells that had invaded the lower surface of the filter were counted by light microscopy and data were expressed as cells/5 high-power fields (HPF)/well.

#### Data Analysis

Data are expressed as means  $\pm$  standard deviation (SD). Student's  $t$  test and one-way analysis of variance (with Bonferroni test for pairwise comparisons) were used for statistical analysis using Prism 2.0 software package from Graphpad Software, Inc (San Diego, CA).

#### Results

##### Constitutive Expression of erbB1 and erbB2 in 4 NSCLC Cell Lines

The NSCLC cell lines used in this study express different levels of erbB1 and erbB2 surface receptors as determined by flow cytometric analysis (Fig 1A). In comparison with the receptor levels expressed in confluent NHBE, H460 cells express low levels of both erbB1 and erbB2; H661 and H358 cells express high levels of erbB2, but not erbB1, whereas H322 cells overexpress both erbB1 and erbB2. Forty-eight-hour exposure of these cells to 80 nmol/L 17AAGA resulted in significant reduction of surface expression of erbB1 or erbB2 in cells exhibiting elevated levels of these receptors. In parallel with inhibition of erbB1 or erbB2 expression, continuous exposure of these cells to 17AAGA for 96 hours resulted in a dose-dependent inhibition of cell proliferation, with estimated IC<sub>50</sub> (concentrations of drug that inhibit 50% of cell proliferation) values ranging from 60 to 90 nmol/L (Fig 1B).

##### Upregulation of E-Cadherin by 17AAGA

Expression of the calcium-dependent epithelial cell adhesion molecule E-cadherin was readily detectable by

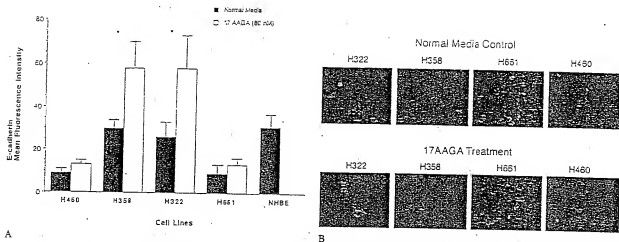


Fig 2. (A) Flow cytometric analysis of surface expression of E-cadherin on NSCLC cells under normal or 17AAGA condition. Similar levels of surface expression of E-cadherin were noted on H322, H358, and normal human bronchial epithelial cells (NHBE). E-cadherin was undetectable on H460 and H661 cells. Significant upregulation of E-cadherin expression was observed in H358 and H322 cells after 48-hour exposure to 17AAGA (80 nmol/L). Data are expressed as means  $\pm$  SD of four independent experiments ( $p < 0.01$ ). (B) Immunofluorescent analysis of lung cancer cells grown in normal media or media containing 17AAGA. After exposure to 17AAGA, H358 and H322 cells exhibit increased membrane localization of E-cadherin.

flow cytometry in NHBE, H322, and H358 cells and not in H460 or H661 cells; results that were consistent with previously published data concerning E-cadherin expression in these cells [11]. The mean fluorescence intensity of E-cadherin in H322 and H358 cells increased 1.5- to 2-fold after treatment with 17AAGA (80 nmol/L for 48 hours) (Fig 2A). The percentages of positively stained cells also increased significantly after 17AAGA treatment in these cell lines ( $63\% \pm 13\%$  and  $58\% \pm 10\%$  of 17AAGA-treated cells vs  $47\% \pm 7\%$  and  $38\% \pm 8\%$  of untreated H358 and H322 cells;  $p = 0.05$  and  $p = 0.02$ , respectively). No upregulation of this adhesion molecule was noted in H460 and H661 cells. Although diffuse cytoplasmic staining for E-cadherin was noted both in control and 17AAGA-treated cells, intense fluorescence indicating localization of E-cadherin to the cell membrane was only observed in treated H358 and H322 cells. No such membrane localization was noted in H460 or H661 cells after 17AAGA treatment (Fig 2B).

#### Downregulation of MMP-9 Secretion

Continuous exposure of NSCLC cells to either 20 or 80 nmol/L of 17AAGA significantly inhibited MMP-9 secretion in a dose-dependent manner, with an overall reduction of up to 50% relative to baseline control levels (Fig 3A). Moreover, incubation of H358 and H322 cells, which express high levels of both erbB1 and erbB2 receptors with agonistic recombinant human EGF or heregulin- $\alpha$ , augmented secretion of MMP-9 by 35% to 50% relative to baseline. 17AAGA treatment completely blocked ligand-mediated upregulation of MMP-9 production in these two cell lines (Fig 3B).

#### Suppression of VEGF Secretion by 17AAGA

NSCLC cells differed widely in their ability to secrete VEGF into the culture media. Values ranged from ap-

proximately 1,000 pg/mL/24 h/ $10^6$  cells (H460, H322) to as high as 15,000 pg/mL/24 h/ $10^6$  cells (H358) (Fig 4A). Cobalt chloride ( $\text{CoCl}_2$ ), which simulates hypoxic conditions by interfering with cellular oxygen sensing mechanisms [14], induced significant upregulation of VEGF secretion (Fig 4A). Forty-eight-hour exposure to 17AAGA (80 nmol/L) significantly suppressed basal VEGF secretion in cultured lung cancer cells, with the magnitude of inhibition ranging from 10% to 50%. Furthermore, 17AAGA suppressed hypoxia-induced upregulation of VEGF secretion; the magnitude of inhibition in treated cells ranged from 30% to 45% relative to untreated "hypoxic" cells. To confirm these findings, cells were incubated in a hypoxic chamber (95%  $\text{N}_2$  and 5%  $\text{O}_2$ ). As expected, profound augmentation of VEGF secretion was noted in response to hypoxia, which could be effectively inhibited by 17AAGA (either 80 or 160 nmol/L) in a dose-dependent manner in all cell lines examined (Fig 4B).

#### Inhibition of Matrigel Membrane Invasion

The ability of cancer cells to migrate through the artificial extracellular matrix membrane, Matrigel, frequently correlates with their invasiveness *in vivo* [16]. Forty-eight-hour exposure to 17AAGA (80 nmol/L) significantly inhibited the ability of H322 and H358 cells to migrate through Matrigel. This inhibitory effect of 17AAGA was profound in H322 cells, with up to a 60% reduction in the number of cells invading through the membrane (109  $\pm$  28 cells/5 HPF of 17AAGA-treated cells vs 293  $\pm$  42 cells/5 HPF in untreated control cells;  $p < 0.001$ ) (Fig 5). 17AAGA inhibited the invasiveness of H358 cells by approximately 40% (116  $\pm$  8 cells/5 HPF of 17AAGA-treated cells vs 190  $\pm$  14 cells/5 HPF of control cells;  $p < 0.01$ ).

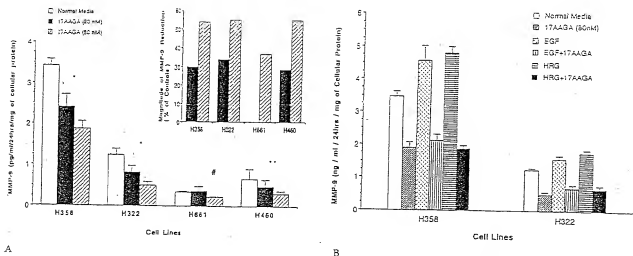


Fig 3. (A) ELISA analysis of MMP-9 secretion by NSCLC cells, grown in the absence or presence of 17AAGA. Exposure of NSCLC cells to 17AAGA resulted in a dose-dependent reduction of MMP-9 levels in the conditioned media ( $P < 0.001$  vs normal media,  $P < 0.01$  17AAGA [80 nmol/L] vs normal media,  $P < 0.05$  17AAGA [80 nmol/L] vs normal media). The magnitude of inhibition of MMP-9 production is displayed in the upper right graph. Results are mean  $\pm$  SD of four independent experiments. (B) Suppression of growth factor-mediated upregulation of MMP-9 secretion by 17AAGA (80 nmol/L) in H322 and H358 cells. Incubating H322 or H358 cells with 20 ng/mL of either epidermal growth factor (EGF) or heregulin  $\alpha$  (Hrg) for 48 hours induced 25% to 40% upregulation of MMP-9 secretion, which was effectively inhibited by 17AAGA. Results are mean  $\pm$  SD of three independent experiments.

## Comment

Better appreciation of the genetic and epigenetic factors that govern malignant transformation and metastasis may facilitate the development of more specific anticancer therapies. In addition to the development of drugs that specifically target the cell cycle machinery,

considerable efforts have been focused on the evaluation of novel biologic or pharmacologic agents that suppress tumor growth or invasion by inhibiting neoangiogenesis or matrix metalloproteinase activity. Activation of erbB protooncogenes profoundly disrupts cell cycle regulation [17], and enhances the expression of "pro-metastasis" phenotypes, namely decreased E-

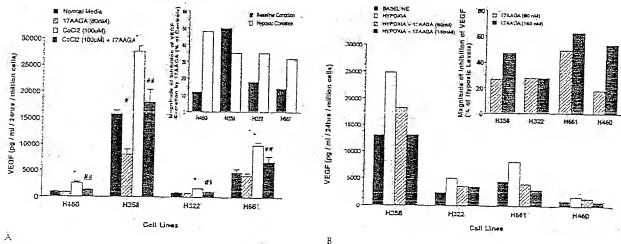


Fig 4. (A) ELISA analysis of basal and hypoxia-stimulated VEGF secretion by 17AAGA. Significant upregulation of VEGF secretion into the culture supernatants by CoCl<sub>2</sub>-stimulated hypoxic condition was observed in all cell lines ( $P < 0.001$ ). 17AAGA at 80 nmol/L substantially suppressed the baseline (in H358 cells) and hypoxia-induced VEGF (in all four cell lines) secretion ( $P < 0.001$  vs baseline controls and  $P < 0.001$  versus hypoxia controls). The magnitude of inhibition of VEGF secretion by 17AAGA (expressed as percentages of baseline or hypoxia controls) is displayed in the upper right graph. Data are mean  $\pm$  SD of three independent experiments. (B) ELISA analysis of VEGF secretion in culture supernatants after physical hypoxia ( $< 5\% O_2$ ) with or without 17AAGA (80 or 160 nmol/L). The magnitude of 17AAGA-mediated inhibition of VEGF secretion (expressed as percentages of hypoxia controls) is displayed in the upper right graph. Data from a representative experiment are shown in this graph.

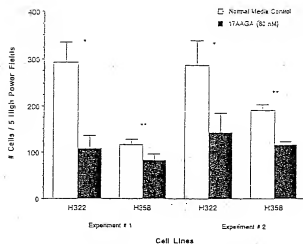


Fig 5. Inhibition of H322 and H358 cell invasion through Matrigel. Exposure of cells to 80 nmol/L 17AAGA for 48 hours reduced their invasion potential by 25% to 50% of that of normal cells. Data are means  $\pm$  SD; each independent experiment was done in quadruplicate (\* $p < 0.005$ , \*\* $p < 0.05$ ).

cadherin expression, increased VEGF and MMP secretion, as well as increased invasion through Matrigel [8–10]. These experimental results correlate closely with observations regarding c-erbB overexpression and aggressive clinical behavior of solid tumors [4–7]. These data provide the rationale for the development of treatment strategies specifically designed to abrogate expression or function of the c-erbB signal transduction pathways. Antagonistic anti-erbB1 and anti-erbB2 monoclonal antibodies, although not always effectively inhibiting tumor cell proliferation, have been shown to significantly upregulate E-cadherin expression, diminish VEGF and MMPs secretion, and reduce chemotaxis through extracellular matrix in vitro [10, 11]. Whereas these monoclonal antibodies may have limited clinical use from technical issues pertaining to delivery of macromolecules to tumor cells, low-molecular weight compounds such as 17AAGA that specifically inhibit erbB-mediated signal transduction pathways at nanomolar concentrations are of particular relevance.

In the present study, we sought to investigate the ability of 17AAGA to modulate expression of E-cadherin, MMP-9, and VEGF, each of which has been implicated in regulation of metastatic potential in tumor cells. The initial step of invasion and metastasis is the dissociation of cancer cells from the primary tumor mass resulting from aberrant expression of a variety of cell surface adhesion molecules, including integrin, immunoglobulins, secretins, and cadherins; the cadherins appear to be critical regulators of this process [18]. Cadherins are calcium-dependent transmembrane glycoproteins that mediate homophilic adhesion between cells. E-cadherin plays an important role in maintaining integrity of epithelial tissue; decreased E-cadherin expression or function correlates with tumor dedifferentiation, increased

invasiveness, and lymph node metastases in a number of human carcinomas including lung, breast, esophagus, and prostate [16, 18]. E-cadherin has been referred to as an “antimetastasis” adhesion molecule, because the high propensity for metastasis formation in E-cadherin-negative cells can be reversed by restoration of expression of this adhesion molecule [19]. Previous studies have indicated an inverse relationship between erbB1 activation and E-cadherin expression in squamous cell cancers of skin and esophagus, as well as breast carcinomas [1, 20]. Upregulation of E-cadherin expression and inhibition of chemotaxis has been observed in H322 cells after exposure to an antagonistic monoclonal antibody directed against the erbB1 receptor [11].

MMPs are enzymes secreted by normal as well as cancer cells that degrade extracellular matrix and influence cell motility, tissue implantation, and angiogenesis. Considerable evidence indicates that MMP expression is increased in cancers relative to adjacent normal tissue, and levels of MMP expression correlate with tumor invasiveness and distant metastases [21]. There exist at least 10 isoforms of these proteases, each of which has unique substrate specificity. Secretion of MMPs is regulated by growth factors, some of which activate erbB1 or erbB2 mitogenic pathways. Inhibition of MMP activity either by recombinant tissue inhibitor of MMP or pharmacologic agents such as batimastat or marimastat decreases tumor growth, invasion, and metastasis in vivo [21]. More interestingly, reduction of MMP levels or inhibition of MMP activity has also been shown to suppress angiogenesis in vitro and in vivo [21].

Tumors smaller than 1 to 2 mm in diameter can receive nutrients by diffusion, but continued growth of the lesions is predicated on neovascularization, which is regulated by complex mechanisms mediated by a balance between proangiogenic and antiangiogenic cytokines present simultaneously in the tumor and normal stromal tissues [22]. VEGF is one of the most potent proangiogenic cytokines, and secretion of VEGF by tumor cells may be sufficient to ensure neovascularization. The extent of neovascularization correlates with aggressive chemical behavior in a variety of malignancies including breast or prostate cancers and melanomas; vascular density and VEGF levels in tumor tissue are significant, independent predictors of systemic recurrence and diminished survival in patients with stage I NSCLC.

Although tumor dissemination, itself, does not require angiogenesis, interruption of angiogenesis should prevent the growth of metastatic deposits [22]. This is the basis of antiangiogenesis therapies using monoclonal antibodies against circulating VEGF or VEGF receptors on endothelial cells. Another strategy to inhibit angiogenesis involves targeting oncogene or tumor suppressor gene mutations directly influencing VEGF transcription through the use of Herceptin in breast cancer patients whose tumors overexpress erbB2, or potentially, by restoration of p53 expression using gene therapy techniques [10, 23]. Our approach



has been to use a pharmacologic agent to achieve similar endpoints while avoiding monoclonal antibodies or cumbersome, inefficient adenoviral vectors that may have limited use in clinical settings. Conceivably, the *in vivo* antiangiogenesis effect of 17AAGA may be more pronounced than anticipated on the basis of our *in vitro* data because this compound suppresses VEGF expression as well as MMP-9, another potent mediator of neoangiogenesis.

Migration through Matrigel depends on a cell's ability to detach from a multicellular aggregate, degrade extracellular matrix, and undergo chemotaxis. The relative contribution of each of these phenotypes to cell invasiveness is not known. 17AAGA-mediated inhibition of cell invasion through Matrigel is the most concrete *in vitro* evidence of the antimetastatic activity of this compound. The mechanisms responsible for 17AAGA-mediated upregulation of E-cadherin, as well as inhibition of tumor-derived VEGF and MMP-9 secretion, most likely depend on the depletion of erbB2 or erbB1 proteins and subsequent decreased mitogenic stimulation via erbB pathways. This notion is supported by the fact that these effects have been observed in cancer cells after treatment with antagonistic monoclonal antibodies against erbB1 and erbB2 receptors [10, 11]. However, additional mechanisms unrelated to inhibition of erbB signal transduction may be relevant given the effects of 17AAGA on a variety of other cell cycle regulatory proteins, including Raf-1 and cyclin-dependent kinase 4 [13].

*In vivo* toxicity profiles of geldanamycin and 17AAGA have been documented in small-animal models; renal and hepatic toxicities are dose-limiting events [24]. However, the nanomolar concentrations required to mediate the "antimetastasis" effects are well below the maximal tolerable doses reported in animal toxicity studies [24]. Underlying mechanisms notwithstanding, our findings extend those pertaining to the antiproliferative effects of 17AAGA. These current data, together with our previous observations concerning the ability of 17AAGA to sensitize NSCLC cells to paclitaxel [25], provide impetus for the evaluation of 17AAGA in lung cancer patients.

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## DISCUSSION

DR ROBERT J. KEENAN (Pittsburgh, PA): One of the things that struck me was the effect or lack of effect on normal human bronchial epithelium. There were at least a couple of cell lines that seemed to have similar erbB-1 and erbB-2 expression to the normals, and yet there was no effect of your treatment on normal tissue. Can you comment on what the potential is for toxicity to normal tissues when there is expression of these genes in normals?

DR NGUYEN: Thank you. I am glad you brought up that issue. In the two graphs that I showed where we used normal human bronchial epithelial cells as a normal control, I did not show the effect of 17AAGA. First of all, I can say that when the normal human bronchial epithelial cells are grown for 96 hours in vitro to reach confluence to mimic the normal condition in vivo, in which there are very few mitotic activities, treating these cells with 17AAGA has no effect on their viability. Treating these cells with 17AAGA indeed does decrease the erbB-1 and erbB-2 expression, but that does not affect cell viability. So we know from our experiments that this drug is not toxic to normal human cells grown to confluence in vitro. I have not tested normal human cells in terms of their ability to secrete MMP-9 or VEGF at all.

DR KEENAN: Sometimes there is differential gene expression in the metastasis compared with the primary tumor. Do you have any data on whether or not some of the antimetastatic effect might be related to those differences in gene expression and whether your treatment might be more or less effective because of that?

DR NGUYEN: That is a very insightful question. No, I have not looked into all the phenotypes. That might contribute to it. The "prometastasis" phenotypes evaluated in this project are those that have been shown to associate with or actively play a role in the process of metastasis formation, but not the metastasis deposit itself.

DR JOHN R. BENFIELD (Los Angeles, CA): Could you tell us a bit more about the cell lines that you used? There is always the problem as to whether cultured cell lines for lung cancer, which, are hard to perpetuate, do in fact represent what happens in lung cancer in humans. What were the cell lines that you used?

DR NGUYEN: The H460 is a large-cell carcinoma, the H322 and H358 cells are bronchoalveolar carcinoma, and H661 is a large-cell carcinoma. So they are all non-small cell lung cancer of different subtypes.

DR BENFIELD: And they have been perpetuated for how many passages? Are these well-established cell lines?

DR NGUYEN: Yes, these cells are well-established, available from ATCC.

DR BENFIELD: Thank you. You alluded to the fact that you are ready now for in vivo testing of the agent. There are at least two ways in which you could do that preclinically. There are hamsters, non-small cell lung cancer models that are rather well established (Benfield JR, Malkinson AM, Schuller HM, Sunday ME. Animal models of lung cancer. In: Kane MA, Bunn PA Jr, eds. *Biology of Lung Cancer*. New York: Marcel Dekker, 1998: 247-93). The other thing that you might want to consider is placing some of the human tumors into nude mice, and then trying the agent while the nude mice are accepting the tumor.

DR NGUYEN: Thank you very much. I appreciate your suggestion.

To be specific about what we are doing, we create either a tumor mass in the flank or actually inject the H358 cells intravenously via lateral tail veins of nude mice to create a metastasis model and then treat the animals at a different time point after tumor cell inoculation with 17AAGA. So this is the experiment that we are carrying out right now.

# Crystal Structure of an Hsp90-Geldanamycin Complex: Targeting of a Protein Chaperone by an Antitumor Agent

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## Summary

The Hsp90 chaperone is required for the activation of several families of eukaryotic protein kinases and nuclear hormone receptors, many of which are proto-oncogenic and play a prominent role in cancer. The geldanamycin antibiotic has antiproliferative and antitumor effects, as it binds to Hsp90, inhibits the Hsp90-mediated conformational maturation/refolding reaction, and results in the degradation of Hsp90 substrates.

The structure of the geldanamycin-binding domain of Hsp90 (residues 9-232) reveals a pronounced pocket, 15 Å deep, that is highly conserved across species. Geldanamycin binds inside this pocket, adopting a compact structure similar to that of a polypeptide chain in a  $\alpha$ -turn conformation. This, and the pocket's similarity to substrate-binding sites, suggest that the pocket binds a portion of the polypeptide substrate and participates in the conformational maturation/refolding reaction.

## Introduction

Geldanamycin, and its closely related analogs herbinone and mabechin, are naturally occurring antitumor antibiotics (DeBoer et al., 1970; Omura et al., 1978; Ono et al., 1982). In the National Cancer Institute's (NCI) *in vitro* screen for antitumor agents, geldanamycin has shown potent activity, achieving 50% growth inhibition ( $IC_{50}$ ) at concentrations as low as 13 nM against the most responsive cell lines, with a mean  $IC_{50}$  of 180 nM against all 60 of the tumor cell lines (Supko et al., 1995). Geldanamycin is active in mouse tumor models as well (Sasaki et al., 1978; Ono et al., 1982), and it has been selected for preclinical development as an antitumor agent by the NCI (Supko et al., 1995).

The antitumor effects of geldanamycin likely result from its ability to deplete cells of two broad classes of growth-regulatory signaling proteins: (1) proto-oncogenic protein kinases, including the erbB2 (Miller et al., 1994; Chavany et al., 1995) and EGF (Murakami et al., 1994) receptor tyrosine kinases, the v-src family of non-receptor tyrosine kinases (June et al., 1990; Xu and Lindquist, 1993; Hartson and Mats, 1994; Whitesell et al., 1994), and the Raf-1 (Schulte et al., 1995; Schneider et al., 1996) and CDK4 Ser/Thr kinases (Stepanova et al., 1996), whose overexpression, or otherwise deregulation, has been observed in diverse human cancers (Bouchard et al., 1989; Hunter and Pines, 1994; Tronick and Aaronson, 1995); and (2) the nuclear hormone receptor family, including the estrogen and androgen hormone receptors (Smith et al., 1995; Nair et al., 1996; Whitesell and Cook, 1996), which can drive the growth of hormone-dependent cancers of the breast (Osborne et al., 1980) and prostate (Isaacs and Coffey, 1979), respectively.

Initially thought to be a nonspecific kinase inhibitor, geldanamycin's target has only recently been identified as the heat shock protein Hsp90 (Whitesell et al., 1994) and its endoplasmic reticulum homolog GP96 (Chavany et al., 1996). In eukaryotes, Hsp90 has dual chaperone functions participating both in the conformational maturation of the nuclear hormone receptors and the aforementioned protein kinases, and in the cellular stress response (Bohen and Yamamoto, 1994; Jakob and Buchner, 1994; Pratt and Welsh, 1994). These two processes are likely to have in common the ability of Hsp90, in cooperation with Hsp70 and other factors, to prevent protein aggregation and mediate the ATP-dependent refolding of heat-denatured proteins *in vitro* and *in vivo* (Freeman and Morimoto, 1996; Schneider et al., 1996).

The best-studied Hsp90-mediated conformational maturation is that of the nuclear hormone receptors, which require the Hsp90 system in order to acquire or maintain a state competent to bind hormone (Bresnick et al., 1989; Picard et al., 1990; Nathan and Lindquist, 1995). In this ATP-dependent reaction, an initial hormone receptor complex that contains Hsp90, Hsp70, and at least two cochaperones, p60 and Hip (p48), is in equilibrium with a complex in which the receptor is in a metastable, nearly mature state competent to bind hormone (Smith et al., 1995; Dittmar et al., 1996). This nearly mature complex lacks Hsp70, p60, and Hip, but contains two new proteins, p23 and one of the three large immunophilins, FKBP52, FKBP54, or CyP40 (Smith et al., 1995; Dittmar et al., 1996). Upon hormone binding, the receptor is released as an active transcription factor (Smith et al., 1995; Dittmar et al., 1996). Treatment with geldanamycin appears to block the conversion to the nearly mature complex, preventing hormone binding and activation, and results in the degradation of the hormone receptor (Whitesell and Cook, 1996).

The requirement for Hsp90 in the activation of the aforementioned protein kinases has been demonstrated both genetically (Xu and Lindquist, 1993; Aigue et al., 1994; Nathan and Lindquist, 1995) and biochemically

<sup>2</sup>These authors contributed equally to this work.

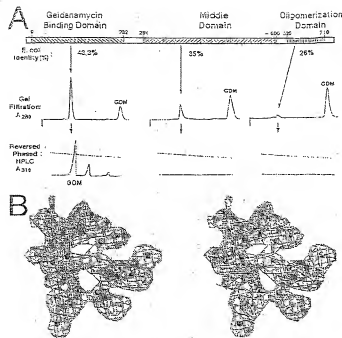


Figure 1. Geldanamycin Binds to the N-Terminal Structural Domain of Hsp90

(A) The three structural domains of Hsp90, identified by proteolytic digestion, are indicated, together with their percent identity in the E. coli homolog. The oligomerization activity at the C-terminal portion has been described (Wearsch and Nicchitta, 1995). The digestion of residues 1-9 is consistent with this region being absent from most Hsp90 sequences except mammalian ones. To identify the geldanamycin-binding domain, the purified structural domains, at 1  $\mu$ M, were incubated with 2.0  $\mu$ M geldanamycin (GIBCO-BRL); each, the free drug was then separated on a gel filtration column, monitoring absorption at 280 nm, and the protein peaks (the C-terminal domain absorbed weakly at 280 nm for lack of tryptophan) were analyzed for the presence of geldanamycin by reverse-phase HPLC, with monitoring at 310 nm. The gel filtration protein peak of the N-terminal domain was the only one that contained geldanamycin; the molar ratio was determined to be approximately 1:1 based on UV absorption. The composition of the two secondary geldanamycin peaks observed in the reversed phase chromatography is not clear, but they could also be produced by the addition of diethylenetriamine in the absence of protein. Geldanamycin is abbreviated as GDM.

(B) Stereo view of the geldanamycin 2Fo-FC electron density before any geldanamycin atoms were built into the Hsp90 model. The map was calculated at 2.0 Å and contoured at 1.0 sigma; also shown is the refined geldanamycin model in a ball-and-stick representation.

(Hartson and Maits, 1994; Chavany et al., 1996; Stepanova et al., 1996). This reaction is not as well understood, but it appears to involve a subset of the molecules found in the complexes with hormone receptors, as well as a protein kinase-specific cofactor, p50<sup>src27</sup> (Whitelaw et al., 1991; Stancato et al., 1993; Cutforth and Rubin, 1994; Stepanova et al., 1996). For some of these kinases, such as Raf-1, Hsp90 association is a prerequisite for their trafficking to the plasma membrane (Schulte et al., 1995); for others, the mechanism of Hsp90-mediated activation is not yet understood. Again, geldanamycin interferes with Hsp90 function and induces the rapid degradation of these kinases by the proteasome system (Murakami et al., 1994; Schulte et al., 1995; Schneider et al., 1996; Stepanova et al., 1996).

In addition to its role in the conformational maturation of signal transduction molecules, the eukaryotic Hsp90 system participates in the refolding of certain thermally denatured polypeptides during the recovery of cells from heat stress (Borkovich et al., 1999). Using firefly luciferase as a model substrate, it was demonstrated that Hsp90 cooperates in this process with Hsp70, Hsp40 (a DnaJ homolog), p50, Hsp, and p23 (Schumacher et al., 1994; Schneider et al., 1996). Thus, the Hsp90 complexes formed with the thermally denatured polypeptide resemble those of hormone receptors and protein kinases. Geldanamycin inhibits the Hsp90-mediated refolding of luciferase, both *in vitro* and *in vivo*, and,

as a result, luciferase is retained in the Hsp90 complex in an unfolded, degradation-sensitive state (Schneider et al., 1996). This shifts the balance from refolding to the proteolytic degradation of the protein (Schneider et al., 1996). Such a mechanism might also explain how, under nonstress conditions, geldanamycin and its homologs cause the degradation of the signaling molecules.

To obtain insights into the function of Hsp90 in these conformational maturation/refolding processes and its inhibition by geldanamycin, we identified the geldanamycin-binding domain of Hsp90 (henceforth Hsp90-GBD), and we determined the crystal structures of this domain and of its complex with geldanamycin.

## Results and Discussion

### Isolation of the Geldanamycin-Binding Domain of Hsp90

To facilitate the structural study of the geldanamycin-Hsp90 interaction, we first used proteolytic digestion and determined that the bovine Hsp90 contains three structural domains (Figure 1A): an N-terminal domain of approximately 25 kDa, a middle domain of approximately 35 kDa, and a C-terminal domain of approximately 10 kDa. We then assayed the three corresponding recombinant domains of human Hsp90 for geldanamycin binding using gel filtration chromatography to remove excess drug and reverse-phase HPLC to detect the bound drug,

Table 1. Summary of Crystallographic Analysis

Diffraction Data and MIR Analysis									
Crystal	Crystal Description	Resolution (Å)	Unique Reflections	Measured Reflections	Coverage (%)	$R_{\text{int}}$ (%)	$R_{\text{meas}}$ (%)	Phasing Power	$R_1$
Native1	I222 (10°C)	2.30	13532	43812	96.0	4.4	—	—	—
Native2	I222 (~150°C)	1.65	34516	361558	98.2	3.8	—	—	—
Native3	P2 <sub>1</sub> -nig (~150°C)	2.20	11381	51794	97.7	3.5	—	—	—
GDM Complex	P2 <sub>1</sub> (~160°C)	1.80	15905	86335	92.2	2.5	—	—	—
K <sub>2</sub> AuCl <sub>4</sub>	I222 (10°C)	2.50	10254	49642	94.6	5.7	17	2.43	0.54
K <sub>2</sub> PtCl <sub>4</sub>	I222 (10°C)	3.20	4713	20129	91.6	5.8	28	1.07	0.53
K <sub>2</sub> PtBr <sub>4</sub>	I222 (10°C)	3.20	4831	23025	93.1	4.7	22	0.75	0.50
Sm(DAC) <sub>3</sub>	I222 (10°C)	3.20	4442	16291	86.0	7.1	30	0.65	0.52

Refinement and Analysis of Atomic Models

Crystal	Resolution (Å)	Reflections (I/I > 2σ)	Atoms Modeled (Protein, Water)	$R_{\text{meas}}/R_{\text{int}}$ (% , %)	rms Deviations (Bonds [Å], Angles [°], B Factor [Å <sup>2</sup> ])
Native1	7.0–2.30	12445	1626, 96	16.9, 25.2	0.012, 1.7, 3.2
Native2	6.0–1.65	32524	1626, 335	19.4, 24.4	0.007, 1.6, 3.3
Native3	7.0–2.20	10845	1675, 246	18.9, —	0.010, 1.7, 3.2
GDM Complex	6.0–1.50	16080	1679, 298	18.9, —	0.009, 1.8, 3.2

Coverage is the percentage measured of the total reflections theoretically observable.  $R_{\text{meas}} = \sum_i |F_o| / \sum_i |F_o|$ , where  $F_o$  and  $F_{\text{calc}}$  are the native and derivative structure factor amplitudes, respectively. Phasing power =  $|F_{\text{calc}}| / |F_{\text{meas}}|$ , where  $F_{\text{meas}}$  and  $F_{\text{calc}}$  are the observed and calculated derivative structure factors, respectively.  $R_1 = \sum_i |F_o - F_{\text{calc}}| / \sum_i |F_o|$ , where  $F_{\text{calc}}$  is the calculated heavy atom structure factor. Figure of merit =  $\langle \sum_i |F_o - F_{\text{calc}}| / \sum_i |F_o| \rangle$ , where  $P(\alpha)$  is the probability distribution for the phase  $\alpha$ . Atoms modeled discounts hydrogen atoms.  $R = \sum_i |F_o - F_{\text{calc}}| / \sum_i |F_o|$ , where  $F_{\text{calc}}$  is the calculated protein structure factor from the atomic model.  $R_{\text{meas}}$  is the R factor calculated using 10% of the reflection data chosen randomly and omitted from the refinement process, whereas  $R_{\text{int}}$  is calculated with the remaining data used in the refinement. rms, bond lengths and angles are the deviations from ideal values; the rms deviation in B factors is calculated between covalently bonded atoms.

and determined that the N-terminal domain, which has the highest sequence conservation among the three domains, binds geldanamycin at an approximately one molar ratio (Figure 1A).

In crystallization experiments, the geldanamycin-binding domain of human Hsp90 (Hsp90-GBD, residues 9–236) produced two distinct crystal forms in which the structure differs by a local conformational change. The geldanamycin-Hsp90-GBD complex crystallized in only one of the two forms. The two apo-Hsp90-GBD structures have been determined at 1.65 and 2.2 Å resolution, respectively, and the structure of the geldanamycin complex at 1.9 Å resolution (Table 1, Figure 1B).

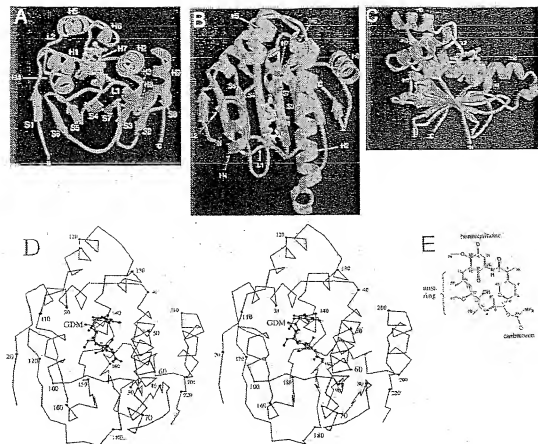
Overall Structure of the Geldanamycin-Hsp90-GBD Complex

The Hsp90-GBD has nine helices and an antiparallel  $\beta$  sheet of eight strands that together fold into an  $\alpha+\beta$  sandwich (Figures 2A–2D). One face of the  $\beta$  sheet is hydrophobic and packs against a layer of five helices; four of these helices (H1, H2, H4, and H9) pack flatly against the  $\beta$  sheet, with their axes parallel to the  $\beta$  strands, while H7 packs against the  $\beta$  sheet at a steeper, almost perpendicular angle. The structure has a second layer of helices that pack on the first layer (H5 and H8) and two smaller helices (H3 and H6) at the periphery of the sandwich (Figures 2A–2D). It is noteworthy that four of the nine helices are of the  $3_1$  type and comprise 11.2% of the amino acids, which is significantly higher than the 3.4% average in the protein database (Barlow and Thornton, 1988). At its center, the helical face of the sandwich has a wide opening that extends into the

hydrophobic core of the structure and results in a pronounced pocket, about 15 Å deep (Figures 2B–2D). The pocket has the  $\beta$  sheet as its base and three helices and a loop as its walls, and contains residues highly conserved across species (Figure 3). The helical face of the sandwich also has a surface groove that leads into the pocket.

In the complex, the 560 Da geldanamycin adopts a compact conformation and binds inside the pocket, filling all but its deepest portion (Figures 2A–2E). The benzoquinone group of geldanamycin binds near the entrance of the pocket, whereas the ansamycin ring, having dimensions similar to those of a five amino acid polypeptide in a turn conformation, inserts into the pocket. There is extensive, though not complete, surface complementarity between geldanamycin and the pocket, and this allows for a high density of van der Waals contacts. Although hydrogen bond contacts are fewer, there being only five of them, one pair from the geldanamycin carbamate group (Figure 2E) can be reasonably described as one of the most important intermolecular interactions in the complex.

The entrance and width of the pocket are likely regulated by a conformational change, as evidenced by structural and positional changes in three helices and a loop in the two different apo-Hsp90-GBD crystal forms (Table 1). One of the conformations, which is essentially identical to that observed in the crystals of the geldanamycin-Hsp90-GBD complex, has a wide enough entrance to allow geldanamycin binding. The other conformation has a narrower entrance, and it would be incompatible with geldanamycin binding.



**Figure 2.** The Hsp90-GBD in an  $\alpha$ - $\beta$  Sandwich with a Deep, Conserved Pocket where Geldanamycin Binds (A-C) Three approximately orthogonal views of the complex. Geldanamycin atoms are colored yellow for carbon, red for oxygen, and blue for nitrogen. In (A), all secondary structure elements are labeled, whereas in (B) and (C), only those discussed in the text are labeled for clarity. Images were prepared with the programs MOLSCRIPT (Kraulis, 1991) and RASTER3D (Meritt and Murphy, 1994). (D) Stereo view of the Ca trace of the complex, with every 10th residue numbered and its Ca atom highlighted as a sphere. (E) Chemical structure of geldanamycin.

#### Architecture and Properties of the Geldanamycin-Binding Pocket

The pocket can be described as a flat-bottomed cone: it is about 15 Å deep, 12 Å in diameter near its entrance, 8 Å midway down, and wide enough at the bottom to hold three water molecules (Figure 4A). The bottom of the pocket is formed by the central portion of the antiparallel  $\beta$  sheet (strands S3, S4, and S7), and the walls by the H2, H4, and H7 helices and the L1 loop (Figure 3). The pocket is deepest by the H7 helix, which packs at an almost perpendicular angle to the  $\beta$  sheet bottom, and is shallowest by the L1 loop. The pocket is of mixed hydrophobic and polar character, with roughly half of the 17 amino acids lining its interior being hydrophobic, a quarter polar, and a quarter charged (these residues are Leu48, Asn51, Asp54, Ala55, Lys58, Ile91, Asp93, Ile96, Gly97, Met98, Asn106, Leu107, Lys112, Gly135, Phe138, Val150, Thr184, and Val186; Figure 3). Although the pocket becomes increasingly hydrophobic toward the bottom, it retains one charged and one polar residue

at its deepest portion (Asp93 and Thr184 from the  $\beta$  sheet).

The Hsp90-GBD domain is highly conserved across species, with 43% of its residues identical in the *Escherichia coli* Hsp90 homolog. The structure reveals that the conserved residues are not distributed homogeneously on the structure, but instead show a striking tendency to cluster in and around the pocket, with 82% of the residues lining the interior of the pocket being invariant from *E. coli* to humans (Figures 3 and 4B). This identifies the pocket as holding the key to understanding the function of this domain. Of particular interest is Asp93, because it is at the pocket bottom in an otherwise mostly hydrophobic environment, and because it is conserved in all known Hsp90 homologs from 35 species.

Supporting a functional significance for this pocket are mutations in the yeast homolog of Hsp90 (Hsp82) that result in either temperature-sensitive or reduced activity phenotypes (Bohen and Yamamoto, 1993; Sullivan and Toft, 1993; Kimura et al., 1994; Nathan and

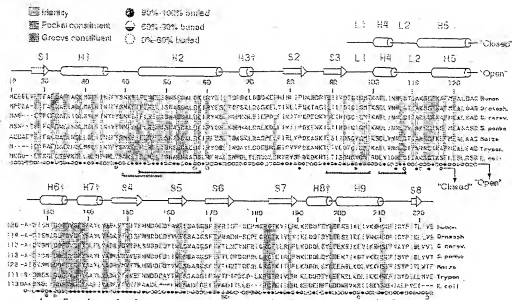


Figure 3. Alignment of Hsp90-GBD Sequences from Several Diverse Species

Secondary structure elements for the P2, [open] form are indicated above the sequence together with the changes observed in the 1222 (closed) form. Invariant residues are highlighted in yellow, residues that make up the pocket are underlined in red, and residues that make up the surface groove are underlined in blue. Residue solvent accessibilities are indicated with circles (open, half-closed, or closed). The dagger symbol indicates 3<sub>10</sub> helices.

Lindquist, 1995], as well as a mutation in the *Drosophila* Hsp90 (Hsp83) that impairs signaling by the Sevenless receptor tyrosine kinase (Cutforth and Rubin, 1994).

These mutations map either in the pocket (Ala55Val, Gly95Ser, Gly183Asp) or in the immediate vicinity of the pocket (Thr36Asn, Ser50Leu, Ala111Thr, Thr115Ile), and

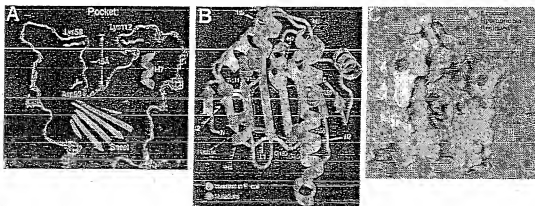


Figure 4. The Hsp90-GBD Has a Conserved Pocket That Is 15 Å Deep

(A) A thin slice of the Hsp90-GBD molecular surface, depicted as a white net, stressing the pocket dimensions. Red indicates surface portions with a negative electrostatic potential, and blue those with a positive potential. The pocket is mostly polar at its entrance (Lys58 and Lys112 are shown), and it becomes predominantly hydrophobic near the bottom, except for Asp83. The approximate locations of the β sheet and of the H7 helix are indicated; the H2 helix would be approximately above the plane of the figure, whereas the H4 helix would be below. This image was prepared with the program GRASP (Nicholls et al., 1991). (B) Yellow highlights the positions of residues invariant in the *E. coli* Hsp90 homolog; magenta highlights residues, shown in space-filling representation, where either temperature-sensitive or inactivating mutations map. (C) Surface representation indicating the pocket and groove. Also indicated, in yellow, are several patches of exposed hydrophobic amino acids in the vicinity of the pocket and groove. These hydrophobic residues are: Phe213 to the right of the groove in this view; Ala117, Ala121, Ala124, Ala126, Met130, and Phe134 to the left of the groove; and Phe20, Ile104, Ala111, and Ile112 to the left of the pocket (prepared with the program GRASP).

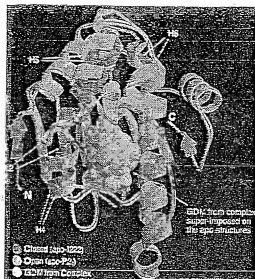


Figure 5. A Conformational Change Alters the Size and Accessibility of the Pocket in the Absence of Geldanamycin

The secondary structural elements that undergo positional and conformational changes are shown in red for the 1222 (closed) and in cyan for the P2, (open) apo-Hsp90-GBD crystal forms. Geldanamycin, in space-filling representation, is superimposed on the figure to stress the effects that the conformational change has on the pocket size.

the structure suggests that most of these mutations would disrupt the structural integrity of the pocket (Figures 3 and 4B).

Leading to the pocket, there is a surface groove formed by the H6 and H5 helices on the sides, and the H2 helix at its bottom (Figure 4C). Its shape is not as pronounced as the pocket, being shallower (about 7–8 Å), broader (about 15 Å), and mostly polar. This groove is conserved, although not as well as the pocket, with 73% of the residues lining its concave surface identical in the *E. coli* homolog (these residues are Asn40, Glu42, Ile43, Arg46, Glu47, Ser50, Ser129, Gln133, His210, Ser211, and Ile214; Figure 3). Since many of these residues are polar or charged, without any significant roles in the structural integrity of this domain, their conservation suggests that the groove may participate in intermolecular interactions important for Hsp90 function.

#### Conformational Change Alters the Size and Accessibility of the Pocket

An approximately 35 amino acid region of the Hsp90-GBD structure, consisting of the H4–L2–H5–H6 secondary structure elements (residues 100–134), has two different conformations, in the two different Apo-Hsp90-GBD crystal forms (Table 1, Figures 3 and 5). We have termed these the "open" and "closed" conformations because they result in different pocket size and accessibility, with only the open conformation being compatible with geldanamycin binding.

At the center of the open-to-closed conformational

change is a displacement of the L2 loop into the pocket by more than 9 Å (Ca–Ca distance), whereupon the L2 loop replaces the H4 helix as one of the pocket walls. The motion of the L2 loop is facilitated by portions of the H4 and H5 helices undergoing helix-to-coil and coil-to-helix transitions, respectively (Figures 3 and 5). The H4 and H5 helices also are displaced, by up to 6 Å. The functional consequence of this conformational change is that the L2 loop acts as a gate that constricts the pocket entrance from a width of 12 Å to 8 Å (Figure 5).

These two alternate conformations have clear electron density in their respective crystal forms and are associated with well-packed side chain arrangements (Figure 3). It is conceivable that this conformational change in vitro may mimic a process that occurs in vivo and may be involved in the regulation or functioning of the pocket. Supporting an in vivo role for the closed conformation is the Ala111Thr mutation on the L2 loop that results in a temperature-sensitive phenotype in yeast (Kimura et al., 1994). This mutation would disrupt the closed conformation structure where Ala111 is fully buried and participates in the hydrophobic packing of the loop, but not the open conformation structure where Ala111 is fully solvent exposed with no apparent structure-stabilizing role.

#### Structure of Geldanamycin

Geldanamycin consists of a closed ansa ring with a planar benzoquinone embedded in it (Figure 2E). The ansa ring is sterically hindered because (1) its backbone consists of a planar amide and three carbon-carbon double bonds (two of them arranged in a 1,3 diene), and (2) of its sixteen backbone atoms, nine carry nonhydrogen substituents: a carbonyl, a carbamate (–OC(O)NH<sub>2</sub>), a hydroxyl, two methoxy, and four methyl groups (Figure 2E).

Hsp90-bound geldanamycin is highly compact and internally well packed (overall dimensions of about 9 × 9 × 9 Å). Its ansa ring is folded over the benzoquinone, forming a C-clamp-like structure, with the benzoquinone forming the top of the (C) and the ansa ring forming the stem and bottom of the (C) (Figure 6A). Two of the methyl groups from the ansa ring are centrally positioned to maximize intramolecular van der Waals contacts: the C25 methyl group from the tip of the ansa ring packs with the benzoquinone, thus bridging the top and bottom halves of the (C), and the C28 methyl group packs with the diene carbon atoms, bridging the two sides of the ansa ring (Figure 6A).

This compact conformation differs from those observed with free geldanamycin (Rinehart and Shield, 1976) or herbimycin (Furusaki et al., 1980), which crystallize from organic solvents in significantly more open, extended conformations. The differences are likely due, in part, to the loss of coplanarity between the amide group and the benzoquinone in Hsp90-bound geldanamycin, as well as to the differences in the crystallization solvents.

#### Geldanamycin–Hsp90 Contacts

The tip of the geldanamycin ansa ring (bottom of [C]), which has the carbamate, C23 methoxy, and C25 and



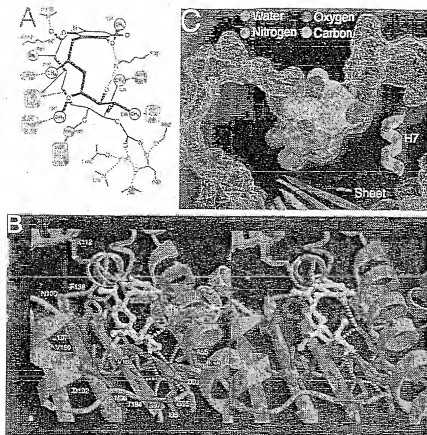


Figure 6. Hsp90-Bound Geldanamycin Adopts a Compact Conformation Reminiscent of the Letter (C) and Binds Hsp90 by Inserting the Tip of Its Ansa Ring inside the Pocket

(A) Diagram of the bound geldanamycin conformation summarizing the hydrogen bond (dotted lines) and van der Waals (arrows) contacts it makes with Hsp90 residues, colored brown. The oxygen atoms of geldanamycin are shown in red, nitrogen atoms in blue, and methyl groups as closed circles; the remaining carbon atoms are not explicitly shown.

(B) Stereo view of the geldanamycin-Hsp90-GBD interactions. Geldanamycin is colored as in Figure 2A, and Hsp90 residues that make up the pocket are colored magenta. Hydrogen bonds are indicated by green dotted lines, and the water molecule that bridges the geldanamycin carbamate group with Asp93 is shown as a red sphere.

(C) Geldanamycin, in space-filling representation, adopts a structure that is, overall, complementary to the pocket, shown as a blue molecular surface net. Although the fit is extensive, there remains a buried cavity at the interface with three trapped water molecules in it (colored magenta), while a fourth water molecule hydrogen bonds with the carbamate group and Asp93. The reduced complementarity near the pocket entrance is evident as gaps between the geldanamycin atoms and the Hsp90-GBD surface. Orientation is similar to that of Figure 4A.

C26 methyl group substituents, binds near the bottom of the Hsp90 pocket and makes a high density of van der Waals contacts, whereas the benzoquinone (top of [C]) is positioned near the entrance of the pocket and makes only a few contacts (Figures 6A and 6B). There is extensive surface complementarity between the compact geldanamycin structure and the pocket, as attested by the fact that a remarkable 85% of the surface area of geldanamycin is buried in the complex (609 Å<sup>2</sup> out of 719 Å<sup>2</sup>).

The most critical portion of the interface is likely to be a hydrogen bond network between the geldanamycin carbamate group and the Asp93 side chain from Hsp90, which are buried at the pocket bottom in an otherwise

mostly hydrophobic environment. The carbamate amino group makes a hydrogen bond to one of the Asp93 side chain oxygen atoms, and its carbonyl oxygen atom makes a water-bridged hydrogen bond with the other Asp93 side chain oxygen atom (Figures 6A and 6B). The Asp93 side chain, which has an identical conformation in the Apo-Hsp90 structures, also hydrogen bonds to the Ser52 OH and the backbone amide of residue 95. This hydrogen bond network is buried about 10 Å away from the nearest solvent surface, and the low dielectric constant of this environment would significantly increase the coulombic attraction due to the partial charges. The importance of this hydrogen bond network is supported by structure activity studies demonstrating

that the removal of the carbamate group, or the attachment of additional atoms to it, completely abolished geldanamycin activity (Schnur et al., 1995b).

At the tip of the ansa ring, the carbamate group contact is flanked by the C23 methoxy and C25 and C26 methyl groups making high density van der Waals contacts with the bottom and sides of the pocket (Figures 6A and 6B). Although the surface complementarity at the pocket bottom is extensive, there remains a buried cavity filled with three water molecules near the C23 methoxy and the carbamate groups (Figure 6C). It is conceivable that the cellular substrates of Hsp90 can fit this cavity as well. Modifications to this portion of geldanamycin, for example by adding to the C23 position a group of 4–6 nonhydrogen atoms, could increase the steric and hydrogen bond complementarity and may yield derivatives with increased affinity for Hsp90.

Halfway between the pocket bottom and the surface, the widening of the pocket causes a decrease in the complementarity and contact density, although the fit is still adequate to exclude bulk solvent (Figure 6C). In this region, the diene and the C27 methoxy groups make van der Waals contacts, and the O5 hydroxyl group makes a hydrogen bond contact with Lys58 (Figures 6A and 6B). As we get closer to the surface, the contact density decreases further; in this region, the C28 methyl group appears to be in an overall unfavorable environment, being within van der Waals contact distance to a backbone carbonyl oxygen atom and a partially buried water molecule (Figures 6A and 6B). The loose fit in this region, both sterically and electrostatically, points to a potential for a significant improvement of the affinity of geldanamycin for Hsp90 (Figure 6C).

At the pocket entrance, the carbonyl oxygen atom of the ansa ring makes a hydrogen bond to a backbone amide (Phe138) at the N-terminal portion of the H7 helix (Figures 6A and 6B). The benzoquinone, which is at the pocket entrance with one face solvent-exposed, makes only a few contacts, and these are primarily solvent-exposed hydrogen bonds made to long lysine chains: one of the benzoquinone oxygen atoms hydrogen bonds with Lys112, and the C29 methoxy group oxygen atom makes a long-distance (3.5 Å) hydrogen bond with Lys58.

#### Geldanamycin Derivatives

Among the naturally occurring homologs, geldanamycin is the most potent. Compared to heribimycin, for example, geldanamycin is more potent by a factor of about four in achieving a 50% reduction ( $IC_{50}$ ) of erbB-2 kinase activity in human SKB-3 breast cancer cells (Miller et al., 1994). The structure suggests that this may be due to the hydrogen bond that the C29 methoxy oxygen atom of geldanamycin makes with Lys58, because heribimycin does not have the C29 methoxy group.

Among the published synthetic derivatives (Schnur et al., 1995a, 1995b), the highest potency, reflecting an improvement in the  $IC_{50}$  of a factor of 4–5, was achieved by an amino group substitution at the C17 position of the benzoquinone (Schnur et al., 1995a). It was proposed that this and several related substitutions at the C17 position improved cellular activity indirectly, by stabilizing the quinone form over the reduced hydroquinone

(Schnur et al., 1995a). The structure supports this hypothesis, as this position is highly solvent exposed in the complex and is a poor candidate for additional Hsp90 contacts.

Overall, however, most modifications decreased or eliminated activity (Schnur et al., 1995a, 1995b). The maintenance of the carbamate group and of the closed, cyclic nature of the ansa ring proved necessary for activity (Schnur et al., 1995b), consistent with the extended hydrogen bond network that the carbamate group makes and with the importance of a closed cyclic ring in limiting conformational flexibility, respectively. Modifications at other positions either had little effect or reduced the activity of geldanamycin in line with space considerations revealed by the crystal structure. For example, additions of small groups to the amide nitrogen and to the C19 position of the benzoquinone, which are juxtaposed in a partially solvent-exposed region of the complex, were tolerated (Schnur et al., 1995a, 1995b), whereas bulky substituents at these positions reduced or eliminated activity (Schnur et al., 1995b).

Our crystal structure suggests modifications, such as those at the C23 methoxy and C22 methyl groups discussed earlier, that could improve the steric and hydrogen bond complementarity between geldanamycin and Hsp90 and could thus provide analogs with increased Hsp90 affinity (a total synthesis of geldanamycin has recently been reported [Miller, 1995]). In addition, by identifying which modifications are compatible with Hsp90 binding, the structure should also help with the improvement of geldanamycin's low plasma half-life (Supko et al., 1995) and other pharmacological properties as well.

#### Implications for Understanding Hsp90 Function

The pocket is best considered as a substrate-binding site, for there are extensive similarities between the pocket and a typical enzyme active site, though no covalent chemistry is implied by the similarities. We note that (1) the pocket has adequate space and hydrophobic content, and is adequately shielded from bulk solvent to tightly bind substrates of a few hundred daltons; (2) there is a nonuniform electrostatic distribution resulting from an aspartic acid at the otherwise hydrophobic bottom of the pocket; (3) the shape and size of the pocket entrance is likely to be regulated by a conformational change; (4) the geldanamycin-binding domain of Hsp90 is the best conserved among its three structural domains, and within this domain, the conserved residues cluster in and around the pocket; (5) mutations that inactivate Hsp90 map in and around the pocket; and, finally, (6) the geldanamycin inhibitor of Hsp90 function binds inside this pocket.

Assuming that the Hsp90 pocket is a substrate binding site, it can then directly follow that its substrate is the protein, or more precisely, a segment of the protein, whose conformational maturation/refolding is mediated by the Hsp90 chaperone system. This pocket-polypeptide association is likely to occur as a late step in the reaction pathway because Hsp70, which is present in all of the early Hsp90 complexes (Smith, 1993; Smith et

al., 1995; Dittmar et al., 1995; Johnson et al., 1996), can bind unfolded proteins (Flynn et al., 1991; Zhu et al., 1995) and is thus the likely initial recruiter of the unfolded polypeptide to the Hsp90 complex. That a polypeptide portion is the substrate for the pocket is supported by the ability of the endoplasmic reticulum homolog, GP96, to associate with a specific viral peptide, though it is not yet known with which domain of GP96 this peptide is associated (Nieland et al., 1996).

Further support is provided by the general similarities between the geldanamycin ansa ring and a peptide. A five amino acid polypeptide in a turn conformation could closely trace the geldanamycin ansa ring backbone, with its amino acid side chains broadly corresponding to the carbamate, methyl, and methoxy groups of geldanamycin (Figure 7). Furthermore, and intended only as an example, steric and hydrogen bond complementarity considerations suggest that a tryptophan amino acid could be accommodated at the polypeptide position corresponding to that of the geldanamycin carbamate group (Figure 7). The tryptophan indole amino group could donate a hydrogen bond to Asp93, in a manner analogous to that of the carbamate amino group, perhaps explaining why Asp93 is conserved, and the hydrophobic portion of the tryptophan could fill the buried cavity that is now occupied by water molecules in the geldanamycin complex (Figure 7). In this region, backbone amide groups, as well as other hydrogen bond donor amino acids, could also satisfy the hydrogen bond potential of Asp93, although they may not have as good of a fit as a tryptophan in the buried cavity.

It is conceivable that the pocket binds a family of related polypeptide sequences, and this could explain the conservation of the pocket residues, because a large family of peptide sequences, in contrast to a single sequence, could not coevolve with amino acid changes in the pocket. This has precedence in the Hsp70 chaperone, which has a peptide-binding channel highly conserved between *E. coli* and humans (Zhu et al., 1996), and binds unfolded proteins by interacting with seven-residue segments, having exposed hydrophobic amino acids at their central positions (Flynn et al., 1991; Zhu et al., 1996).

A model in which the polypeptide substrate binds in the pocket along the reaction pathway would be supported by the observation that geldanamycin causes the dissociation of the *v-src*-Hsp90 complex in transformed cells (Whitesell et al., 1994). Also consistent are observations from the *in vitro* conformational maturation of the steroid receptors. In this reaction, a transient complex of the receptor that includes Hsp90, Hsp70, and several other cofactors is in equilibrium with the hormone-responsive state of the receptor, which is bound to Hsp90, p23, and an immunophilin (Smith et al., 1995; Dittmar et al., 1996; Nair et al., 1996). Geldanamycin blocks the formation of the latter complex, consistent with receptor-pocket interactions being required to either arrive at or to maintain the hormone-responsive state of the receptor (Smith et al., 1995; Nair et al., 1996). In principle, the interruption of this reaction by geldanamycin can be interpreted in other ways, for example, to mean that geldanamycin directly competes with p23 (Smith et al., 1995) binding to the pocket. This

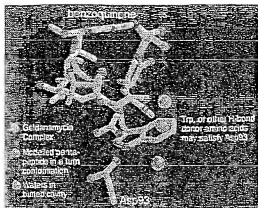


Figure 7. The Geldanamycin Ansa Ring Adopts a Structure Reminiscent of a Pentapeptide in a Turn Conformation. Geldanamycin and Asp93 of Hsp90 are in yellow, the buried water molecules at the geldanamycin-Hsp90-GBD interface are in magenta, and the modeled polypeptide is in cyan. Red and blue indicate oxygen and nitrogen atoms, respectively. A tryptophan at the peptide position corresponding to that of the carbamate group in geldanamycin could donate a hydrogen bond to Asp93 of Hsp90 and also pack in the hydrophobic cavity now occupied by the three water molecules. Backbone amide groups and several other amino acids could also hydrogen bond to Asp93, but their fit in the pocket would not be as good as that of a Trp.

is possible; however, we note that: (1) it is unlikely that such an unusual pocket would have evolved to bind a protein cofactor; (2) p23 and the pocket could have coevolved resulting in far lesser sequence conservation at the pocket; and (3) *E. coli* and lower eukaryotes do not seem to have p23, at least not one that is as conserved as the Hsp90-GBD.

On the other hand, the isolation of complexes of heat-denatured luciferase and of Raf with Hsp90 in the presence of geldanamycin (Schneider et al., 1996) at first appears to be inconsistent with the pocket binding these substrates. It is conceivable, however, that Hsp90 contains additional polypeptide-interacting sites that are not blocked by geldanamycin and that act at an earlier stage. These additional sites can be either within its geldanamycin-binding domain (for example, the conserved surface groove) or in the rest of the 732 residue protein. Alternatively, the observed association of geldanamycin-bound Hsp90 with the protein substrate may be indirect, since these complexes contain several other protein cofactors, such as Hsp70, which may contribute to polypeptide binding (Bose et al., 1996; Duina et al., 1996; Freeman et al., 1996; Schneider et al., 1996). In any case, geldanamycin blocks the refolding of the heat-denatured Raf and luciferase substrates, and this ultimately results in their degradation (Schneider et al., 1996).

The crystal structure of the Hsp90-GBD reveals a pocket whose properties indicate that it is a likely binding site for the polypeptide substrate. That geldanamycin, by binding in this pocket, inhibits the Hsp90-catalyzed conformational reaction both *in vitro* and *in*

vivo strongly argues that the pocket-substrate interaction is a required step in this reaction. The crystal structure of this pocket, as well as that of the geldanamycin-pocket complex, also provides a rational basis for the development of geldanamycin derivatives with improved antitumor activity.

#### Experimental Procedures

##### Identification of the Geldanamycin-Binding Domain of Hsp90

Bovine Hsp90, prepared from brains as described (Koyasu et al., 1986), was digested by subtilisin, and the products were sorted by N-terminal sequencing and mass spectroscopy. The C terminus of the middle domain is estimated based on its mobility in SDS-PAGE because this fragment was not amenable to mass spectroscopic analyses. The corresponding fragments of human Hsp90 were overexpressed in *E. coli* using the pET3d vector (Novagen), and the recombinant domains were purified by ion exchange and gel filtration chromatography.

##### Crystallization and Data Collection

For crystallization, the recombinant human Hsp90-GBD (residues 9–236) was concentrated by ultrafiltration to 30 mg/ml and was further purified by gel filtration chromatography. The geldanamycin-Hsp90-GBD complex was prepared by mixing the purified protein with a 2-fold molar excess of geldanamycin (GIBCO-BRL) fractionation by gel filtration, and concentration by ultrafiltration to 17 mg/ml. Two crystal forms were grown at 7°C using the method of hanging-drop vapor diffusion. One form (Native1 and Native2 in Table 1) grew from a buffer of 0.1 M sodium cacodylate (pH 6.5), 0.2 M ammonium sulfate, and 30 percent w/v polyethylene glycol 8000 (PEG8000) in spacegroup P2, with  $a = 65.2$  Å,  $b = 90.1$  Å,  $c = 101.2$  Å. Another form (Native3) grew from 0.1 M Tris-HCl, 0.2 M magnesium chloride, and 30% PEG4000 (pH 8.5) in spacegroup P2, with  $a = 53.4$  Å,  $b = 44.1$  Å,  $c = 54.0$  Å,  $\beta = 115.7^\circ$ . Crystals of the geldanamycin-Hsp90-GBD complex (GDM complex in Table I) grew from a buffer of 0.1 M Tris-HCl, 0.2 M sodium acetate, and 33% w/v PEG4000 (pH 8.5) in spacegroup P2, with  $a = 53.7$  Å,  $b = 44.3$  Å,  $c = 54.6$  Å,  $\beta = 116.1^\circ$  (same space group as Native3), and had a violet coloring. Data were collected on an R-AXISRIC imaging plate detector mounted on a Rigaku 200kH X-ray generator, at 10°C. For Native1 and the dehydrated crystals. For the other data sets, crystals were flash frozen at  $-160^\circ\text{C}$  either in 30% PEG8000 and 20% glycerol (Native2) or in 35% PEG4000 (Native3 and GDM complex).

##### Structure Determination and Refinement

The heavy atom derivatives were prepared by soaking 1222 form crystals in a buffer of 75 mM sodium cacodylate, 0.15 M ammonium sulfate, and 40% PEG8000 (pH 6.5) containing one of the following heavy atom solutions: saturated  $\text{K}_2\text{AuCl}_4$  for 52 hr, 20 mM  $\text{K}_2\text{PtCl}_6$  for 24 hr, and 20 mM  $\text{K}_2\text{PbI}_4$  for 60 hr. An additional derivative was obtained in 5 mM sodium cacodylate, 250 mM NaCl, 33% PEG8000 with 0.5 mM  $\text{Sm}(\text{DMSO})_3$  (pH 7.0) for 14 hr. The multiple isomorphous replacement (MIR) analysis was performed using the CCP4 suite of programs (Collaborative Computational Project, Number 4, 1994). MIR phases had a mean figure of merit of 0.65 to 3.2 Å and were improved by solvent flattening and histogram matching. The initial model building was followed by several cycles of refinement with the program X-PLOR (Brunger, 1991) and by weighted combination between model phases and MIR phases, and this allowed for a continuous trace of residues 11–223. Amino acids 9–16 and 224–236 in the 1222 form were not visible in the electron density maps and are presumed to be disordered. The P2 crystal form was solved by molecular replacement with X-PLOR. Difference density maps revealed that amino acids 165–132, and especially 105–112, had undergone significant structural rearrangement compared to the 1222 form. In the crystals of the complex, geldanamycin was prominent in the early difference electron density maps and was built using the crystal structure of free geldanamycin (Purtschke et al., 1990) as a starting point. In addition, in both P2 forms, the amino-terminal

residues 11–16 were visible and modeled, whereas two solvent-exposed loops, 121–130 and 212–214, became partially disordered relative to the 1222 crystals. Residues in all models were in the allowed regions of the Ramachandran plot, with 85% or more in the most favored regions. It is not clear what causes the conformational change in vitro; the L2 loop, which changes the most, is not involved in any significant crystal-packing contacts, though the H5 and H6 helices are in both crystal forms. In addition to magnesium and geldanamycin, the growth of the P2 form can be induced by acidic pH as well.

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### **Related Proceedings Appendix**

Decision in Appeal No. 2006-1468 dated August 28, 2006.

The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

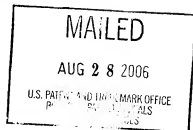
**UNITED STATES PATENT AND TRADEMARK OFFICE**

**BEFORE THE BOARD OF PATENT APPEALS  
AND INTERFERENCES**

Ex parte NEAL ROSEN, SCOTT D. KUDUK,  
SAMUEL J. DANISHEFSKY, FURZHONG F. ZHENG,  
LAURA SEPP-LORENZINO, and OUATEK OUERFELLI

Appeal No. 2006-1468  
Application No. 09/937,192

HEARD: JULY 11, 2006



Before ADAMS, GRIMES, and GREEN, Administrative Patent Judges.

GREEN, Administrative Patent Judge.

**DECISION ON APPEAL**

This is a decision on appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 3, 4, 6 and 9-34. Claims 3, 12 and 13 are representative of the claims on appeal, and read as follows:

3. A chemical compound comprising first and second hsp-binding moieties which bind to the pocket of hsp90 with which ansamycin antibiotics bind, said binding moieties being connected to one another by a linker, wherein the first and second hsp-binding moieties are each an ansamycin antibiotic and retain the ability in the chemical compound to bind to the pocket of hsp90.



12. A method for destruction of cells expressing a HER-family tyrosine kinase, comprising administering to the cells a chemical compound comprising first and second hsp-binding moieties which bind to the pocket of hsp90 with which ansamycin antibiotics bind, said binding moieties being connected to one another by a linker, wherein the first and second hsp-binding moieties are each an ansamycin antibiotic and retain the ability in the chemical compound to bind to the pocket of hsp90.
13. A method for treating cancer in a patient suffering from cancer, comprising administering to the patient a therapeutic composition comprising a chemical compound comprising first and second hsp-binding moieties which bind to the pocket of hsp90 with which ansamycin antibiotics bind, said binding moieties being connected to one another by a linker, wherein the first and second hsp-binding moieties are each an ansamycin antibiotic and retain the ability in the chemical compound to bind to the pocket of hsp90.

Claims 12-30 and 32-34 stand rejected under 35 U.S.C. § 112, first paragraph, on the grounds that the specification does not enable the full scope of the claimed subject matter. In addition, claims 3, 4, 6 and 9-34 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Finally, 3, 4, 6 and 9-34 stand provisionally rejected under the judicially created doctrine of obviousness-type patenting as being unpatentable over claims 1, 2, 6, 7, 12, 13 and 15-40 of copending application 09/960,665. As appellants do not argue the merits of the provisional obviousness-type double patenting rejection, we summarily affirm that rejection. After careful review of the record and consideration of the rejections under 35 U.S.C. § 112, first and second paragraphs, we reverse.

DISCUSSION

Claims 12-30 and 32-34 stand rejected under 35 U.S.C. § 112, first paragraph,

because the specification, while being enabling as a method of treating HER-2 expressing cancer using geldanamycin dimer linked by a 4-carbon chain at the 17-positions of each, does not reasonably provide enablement for treating any and all cancers and for destruction of other cells or treating cancers generally using "a chemical compound comprising first and second hsp-binding moieties which bind to the pocket of hsp90 with which ansamycin antibiotics bind, said binding moieties being connected to one another by a linker, wherein the first and second binding moieties are each an ansamycin antibiotic and retain the ability in the chemical compound to bind to the pocket of hsp90."

Examiner's Answer, page 3.

"[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." In re Marzocchi, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971) (emphasis in original). "[I]t is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." Id. at 224, 169 USPQ at 370. Here, the examiner has not provided

"acceptable evidence or reasoning which is inconsistent" with the specification, and therefore has not met the initial burden of showing nonenablement.

While the examiner engages in a Wands analysis, see In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1403 (Fed. Cir. 1988) (noting that facts that should be considered in determining whether a specification is enabling include: (1) the quantity of experimentation necessary to practice the invention, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims), the examiner's primary concern appears to be that "[t]here is no general treatment for cancer and there is no correlation between the assays relied upon by applicants and the ability to treat all cancers." Examiner's Answer, page 4. The examiner states:

The claims are drawn to disorders that are not related and whose treatment using a single compound is unknown. Pancreatic cancer, for example, has proven extremely difficult to treat. Gastric cancer embraces several different types of cancers which includes, Adenocarcinomas (cancers started in the gland cells in the stomach lining), Squamous cells cancers are cancers in the skin-like cells that are mixed with gland cells to make the stomach lining, Lymphomas, sarcomas (cancer that begins in the muscle layer of the stomach is sarcoma) and Neuroendocrine tumours (cancers that grow in hormone producing tissues, usually in the digestive system). Treatment for each is different.

Id. at 6. The examiner, however, provides no evidence to support the above.<sup>1</sup>

The examiner also asserts that there is no predictability because the invention is pharmaceutical in nature, and that "the amount of guidance presented in the specification as to which compounds are sufficiently active to be useful for the claimed uses is nonexistent." Id. at 5. Again, the examiner has provided no evidence or scientific reasoning to support those assertions, and thus has not met his burden in demonstrating that the specification fails to enable the full scope of the claimed subject matter. In addition, the treatment of "any and all cancers" is not the proper standard as a claim may encompass inoperative embodiments and still meet the enablement requirement of 35 U.S.C. § 112, first paragraph. See Atlas Powder Co. v. E.I. Du Pont De Nemours & Co., 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984), In re Angstadt, 537 F.2d 498, 504, 190 USPQ 214, 218 (CCPA 1976), In re Cook, 439 F.2d 730, 732, 169 USPQ 298, 300 (CCPA 1971).

Moreover, appellants have provided evidence demonstrating that a monomeric ansamycin compound, as well as other hsp90 inhibitors "are efficacious in a variety of tumor types including breast cancer, ovarian cancer, pancreatic cancer and gastric cancer . . . , other HER kinase overexpressing tumors, and tumors which do not over express HER kinase." See Appeal Brief,

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<sup>1</sup> The examiner does cite Sreedhar et al., Biochimica et Biophysica Acta, pp. 233-242 (2004). Appellants state in the Reply Brief that the Sreedhar reference is not of record, and we also could not find it in our review of the record. In response, the examiner merely stated that "[t]he reply brief . . . has been entered and considered." Paper mailed February 17, 2006. Thus, we have not considered the examiner's arguments to the extent that they rely upon the Sreedhar reference.

page 9, as well as the references cited by Appellants on that page. Again, the examiner has not brought in any evidence or provided any scientific reasoning to refute the evidence provided by appellants.

Therefore, as the examiner has failed to set forth a prima facie case of unpatentability under 35 U.S.C. § 112, first paragraph, we are compelled to reverse the rejection.

Claims 3, 4, 6 and 9-34 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter that appellant regards as the invention. See examiner's Answer, page 7.<sup>2</sup>

The examiner's first concern is that "[t]he metes and bounds of . . . 'ansamycin' are unknown." As noted by appellants, however, "[t]he term 'ansamycin antibiotic' is a recognized term of art," see Appeal Brief, page 12,<sup>3</sup> and the examiner has provided no evidence to demonstrate that there is confusion in the art regarding that term.

The examiner's second concern is that the nature of the linker is unknown. See Examiner's Answer, page 7. As noted by appellants, see Appeal Brief, page 14, the specification at page 4 and page 6, describe the linker, and how activity varies with linker length. Thus, we find that one skilled in the art

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<sup>2</sup> The examiner designates this rejection as a "New Grounds of Rejection." See Examiner's Answer, page 7. As noted by appellants, however, see Reply Brief, page 1, the rejection appeared in the non-final rejection mailed July 11, 2005.

<sup>3</sup> See also Rinehart, "Antibiotics with Ansa Rings," Accounts of Chemical Research, Vol. 5, pp. 57-64 (1972), attached to the Appeal Brief, which discusses the then new class of ansamycin antibiotics.

would understand the metes and bonds of the term linker. See Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1385, 231 USPQ 81, 94-95 (Fed. Cir. 1987) (noting that claims are in compliance with 35 U.S.C. § 112, second paragraph, if “the claims, read in light of the specification, reasonably apprise those skilled in the art and are as precise as the subject matter permits.”).

The examiner’s third concern is with the term bind. According to the examiner, “[t]he term ‘bind’ in the claims is indefinite. There is no way of knowing whether a given compound would bind. Binding is a process which cannot be observed, merely inferred, which is unreliable.” Examiner’s Answer, page 8. We cannot understand the examiner’s concern in this regard, however, because “bind” is an art recognized term, and it is unclear if “bind” is unacceptable, what term would be acceptable to the examiner.

Finally, the examiner objects to the term “retain the ability to bind,” asserting that it is unclear. According to the examiner:

Does it mean that the compound is supposed to bind exactly as strongly as the monomer binds or does it mean that it simply needs to bind? Both definitions could be valid, but Appellants need to clarify whether the term “retain” means as strongly or weakly as the monomer or simply the ability with no regard as to how it accomplishes the binding.

Id.

From the examiner’s statement that “[b]oth definitions” he proposes are valid, the examiner understands what is meant by the phrase “retain the ability to bind.” The examiner’s concern thus appears to be the breadth, however, “breadth is not to be equated with indefiniteness.” In re Miller, 441 F.2d 689,

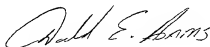
693, 169 USPQ 597, 600 (CCPA 1971); see also In re Hyatt, 708 F.2d 712, 714-15, 218 USPQ 195, 197 (Fed. Cir. 1983).

For the reasons set forth above, the examiner has failed to set forth a prima facie case of unpatentability under 35 U.S.C. § 112, second paragraph, is reversed.

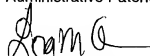
CONCLUSION

Because the examiner has failed to set forth a prima facie case of unpatentability under 35 U.S.C. § 112, first or second paragraph, the rejections under those sections of the statute are reversed. Because appellants do not argue the merits of the provisional obviousness-type double patenting rejection, we summarily affirm that rejection.

AFFIRMED

  
Donald E. Adams  
Administrative Patent Judge

  
Eric Grimes  
Administrative Patent Judge

  
Lora M. Green  
Administrative Patent Judge

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) BOARD OF PATENT  
) APPEALS AND  
) INTERFERENCES  
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